



# **LIFE SCIENCE IN MODERN PERSPECTIVES**

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### **Foreword**

It is, indeed, a great effort on the part of UGC – Academic Staff College and the Department of Physiology, University of Calcutta, to publish the book *Life Science in Modern Perspectives*. The publication is a compendium based on the presentations, discussion and interaction held in the refresher course covering many emerging areas of Life Science. I am impressed to find that the view-points that have emerged in this collection are highly interesting and informative. The areas covered range from life on the planet, DNA double helix, genomics and proteomics on one end and the problems related to various diseases that affect human health severely on the other. In addition, the classical and current topics and their different aspects have been adequately discussed.

The editors, Prof. (Mrs.) Pratima Chatterjee and Dr. Amar K Chandra, have given due importance to issues like drug toxicity, drug-nutrient interaction, micronutrient deficiency and occupational hazards which are most relevant for human health in the present days. In the same context management of diseases has been accorded due emphasis. Similarly, vaccination, herbal medicine and management of body profiles have been discussed in detail. Other important features of this book are inclusion of recent topics like bio-ethics, reproductive toxicology, lung-function, neurogeneration and neuroprotection, genetic drift, photosynthesis regulation, oxidative stress and membrane channels. Discussion of application of instrumental techniques like stereotaxic techniques, electrophysiological techniques and biotechnology will add to the appeal of the book.

I congratulate the editors of this book, Prof. (Mrs.) Pratima Chatterjee and Dr. Amar K Chandra, for this laudable endeavour.

**Asis Kumar Banerjee**



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*From the Desk of the Honorary Director*

The UGC-Academic Staff College, University of Calcutta, has an active programme of publishing quality books based on the study materials presented in Refresher Courses in different subjects. A Refresher Course provides a rare occasion of interaction of the participants with many eminent persons in an area, and publication of a book containing the lecture materials is essential for dissemination of knowledge and thought to a broad spectrum of persons, including senior students, interested in the subject.

Life Science is one of the most challenging interdisciplinary areas of the modern time. Prof. Pratima Chatterjee and Dr. A. K. Chandra have done a wonderful job by collecting and editing a number of illuminating and exciting articles contributed by the resource persons. I am sure people at large connected with teaching and research in this area will find *Life Science in Modern Perspectives* useful to get an overview of the present scenario of quite a few aspects of Life Science.

**Binay K. Dutta**  
Honorary Director

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From the desk of the Honorary Director

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## Preface

Exploration of the physiochemical basis of life process has been a challenging issue and scientists from diverse disciplines have joined their hands and mind to resolve it. Keeping this spirit in mind teachers and researchers of different backgrounds were invited to this Refresher Course in Life Science to create a unique platform for deliberation the issues related to the current achievements of the subject. Spectacular advances in the field of life science have been noted in the last few decades starting from three-dimensional DNA structure to genomics and proteomics through innumerable studies and techniques. Life is now viewed through a molecular basis replacing many of the old ideas. Studies are in progress to find out etiology of different deadly diseases and their possible management through biotechnological means, drug-nutrient interactions, genomic vaccination, neuroprotection, genetic drift, DNA dynamicity, nanotechnology, apoptosis, oxidative stress, toxicology, micronutrient deficiency and many other thrust areas aiming at alleviating human sufferings.

In this book both the basic and applied aspects of contemporary research in life science discussed by the scientists actively engaged in the respective fields have been put together. The publication is expected to refresh the students and researchers of different branches of classical bioscience. We are indebted to our Honb'le Vice-Chancellor Prof. Ashish K. Banerjee for writing the foreword of this book. The authors of the article are gratefully acknowledged for their valuable contributions. Sincere thanks and gratitude to Prof. S. Das, Pro-Vice Chancellor (Academic), University of Calcutta and Prof. T.K. Mukherjee, Pro-Vice Chancellor (Business Affairs & Finance), University of Calcutta for their kind encouragement and support for this endeavour. Thanks are due to Dr. Shymal Roy Choudhury for his assistance in proof reading of this publication. We are indebted to Prof. B K Dutta, Honorary Director, UGC Academic Staff College, University of Calcutta for his sincere approach to prepare a manuscript based on the discussed topics on the very first day of the Refresher Course and continuous encouragement and suggestion till the day of publication of this book. Special thanks are due to Prof. P N Ghosh, Dean of the Faculty of Science, Prof. A. Gomes, Head of the Department of Physiology, Prof. A. K. Chatterjee, Prof. (Mrs.) M Bal and Prof. A.M. Chandra of the Physiology Department for their interest and help in this endeavour.

Department of Physiology  
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Dolpurnima, 2004

Pratima Chatterjee  
Amar K Chandra



# The Story of DNA Double Helix

PROF. MANJUSRI BAL

**A**ll of us, who are interested in the functioning of life-processes, are familiar with DNA, the majestic macromolecule, the blue print of life, the carrier of hereditary information, the Mona Lisa of modern science.

Fifty years back two young scientists, James Dewey Watson (American Biologist & Microbial geneticist) and Harry Crompton Crick (English Physicist) from Cavendish Laboratory, University of Cambridge, England, proposed the double helix structure of DNA, which was published in the British scientific journal "Nature" on April 25<sup>th</sup>, 1953. Their seminal discovery completely revolutionized biology, a new understanding was born, a total conceptual change resulted, which triggered the era of Modern Biology.

The discovery of the double helix had such tremendous immediate as well as far reaching impacts that it may be termed the most significant scientific discovery of the 20<sup>th</sup> century. In scientific circles, the DNA double helix has brought together Physicists, Crystallographers, Chemists, Biochemists, Geneticists, Physiologists, Botanists, Zoologists together around a new identity: "Molecular Biology". This discovery has directed the development of a new industry - "Biotechnology" and influenced Medicine and Agriculture to a great extent. Today the DNA double helix serves as a unifying symbol and provides a collective identity for many different areas of science and thus has become an "icon" of modern science.

In this article, I would like to present a glimpse of how this all began and how Watson and Crick, finally established the double helical structure of DNA.

It was Johann Friedrich Miescher, the Swiss biochemist who first noted DNA in cell nucleus in 1869. Miescher was studying the chemistry of cellular structure, under Felix Hoppe Seyler (1825 - 1895) in Tübingen, Germany. While studying cell nucleus, he isolated leucocytes from the used bandages of the surgery department of the nearby University Hospital of Tübingen. During this process of isolation, the cells swelled and gave rise to a highly viscous porridge that was impossible to handle. Using dilute alkali, Miescher observed that something having a fine, thread-like appearance could be extracted from this swollen-cell-porridge that gets precipitated when neutralized with acid. He felt sure that this unknown material was derived from nucleus. After a lot of hard work he developed a new method and prepared undamaged nuclei, free of cytoplasm. He isolated the same thread-like precipitate from these nuclei. He analyzed and realised that he had discovered an entirely new group of substance. He named this

new material " **Nuclein** ". His findings were reported in Hoppe-Seyler: Medicinisch-Chemische Untersuchungen (Hoppe-Seyler's Medico-Chemical Investigations) in the spring of 1871. He repeated his work after returning to his home town, Basel, using Salmon-Sperm as a source of nuclein. Here, he worked in extremely primitive conditions. In one of his famous letters he mentioned to his uncle Wilhelm His, that *"In order to prepare nuclein, I go to the laboratory at 5 o'clock in the morning and work in a unheated room. No solution may be kept for more than five minutes, and no precipitate left for more than an hour, before it is all preserved under absolute alcohol. The work often goes on until late at night"*. The history of science owes much to people like Miescher who have worked under the most appalling conditions, without any economic incentive and made incredible discoveries.

In 1872, Meischer presented a paper on nuclein at a meeting of the Society for Scientific Research in Basel. In 1874, he published a full account of his work under a somewhat misleading title " The Spertmatozoa of some vertebrates ". This article appeared in the journal of the "Transactions of the Society for Scientific Research" of Basel. Unfortunately, Meischer's results were questioned, mistrusted and brutally rejected as "nothing but an impure albuminous substance". Scientists from Germany, England, France dismissed Meischer's nuclein as a phosphorylated protein. The lack of appreciation and the harsh criticism from his scientific peers must have hurt him deeply. He gave up his research in nuclein and concentrated on physiological research. He worked on physiology of the spawning salmon, later on, on the physiology of respiration and also on nutritional status problems. Meischer was a very modest person, intelligent, shy, introspective and conscientious of his duties. He was not an ambitious man, neither did he long for fame and recognition. He considered his research as paramount and carried it out with religious conviction.

Meischer was born on August 13<sup>th</sup>, 1844 in Basel. He died untimely, unheard and unsung on August 26, 1895 in a sanatorium at Davos of tuberculosis of lungs. Nevertheless, the credit for discovery of DNA belongs to Friedrich Miescher, the least pretentious of scientific heroes. One person however had realized his contribution, the famous physiologist Karl Ludwig, Miescher's former professor in Leipzig. In a moving letter to Meischer, just before his death, paying tribute to Meischer's discovery of nucieln, Ludwig wrote *"When in coming centuries, men work on the cell, your name will be gratefully remembered as a pioneer in this field"*.

Meischer had made a landmark discovery. Surely, if his discovery of nuclein had been unanimously hailed as the great step forward in biomedicine that it eventually proved to be, he would not have changed his field. May be the story of DNA would have been written in a different fashion and at least fifty years earlier. The cold reception of nuclein by the scientific community not only depressed Miescher but also took DNA research,back by a few decades. In the mean time, during the 2<sup>nd</sup> half of 19<sup>th</sup> century, Germany began industrial production of new synthetic dyes, which not only provided economic upliftment and increased political power to German empire, but also contributed to the development of cell-biology. These new dyes were utilized by German-biologists for staining cells, and ideas regarding "chromatin" (Walter Fleming, 1882) "chromosome" i.e., coloured body (Edouard Van Beneden, 1883 ) emerged. The characteristic behaviour of the chromosome during cell division, their longitudinal

cleavage and the equal distribution of the replicated chromosomes among the daughter cells led Wilhelm Roux (1885) to postulate that chromosomes are the bearer of heredity. Richard Altman, a German scientist developed a method for preparation of protein free nuclein in 1889 and it was found that nuclein is acidic in nature. So in 1889, Altman renamed nuclein as **nucleic acid**. Eminent biologist like August Weismann, Oscar Hertwig and others suggested and finally the American biologist Edmund B Wilson in 1896 concluded that chromatin is nuclein. The American geneticist Walter Sutton in 1903 and the German cytologist Theodore Boveri in 1904 stated clearly that chromosomes are real carriers of heredity. By the dawn of the 20<sup>th</sup> century, the idea that dominated biology was that chromatin, that is nuclein or nucleic acid is the genetic material. Unfortunately, Miescher never returned to this field and never thought that his "nuclein" could have a role in heredity. In the early 20<sup>th</sup> century, many scientists from central Europe and USA started analysing nucleic acid. August Weismann (1834 - 1914), Adolf Von Baeyer (1835 -1917), Emil Fischer (1852 -1919), Albrecht Kossel (1853 - 1927), were some of the important contributors, who established the composition of nucleic acid. It was gradually established that there are two kinds of nucleic acids: Deoxyribonucleic Acid (DNA) and Ribonucleic Acid (RNA). Each chromosome consists of very long strands of DNA, coiled and folded along with proteins, to produce the compact body of chromosomes. But even after the chemical nature of chromosomes was revealed, it was not immediately clear how they function as carriers of heredity.

Meanwhile, the concept of gene was emerging. Gregor Johan Mendel a Moravian monk in 1865 first proposed a scientific theory of heredity. Based on his classic experiments with pea-plants, he proposed that in sexually reproducing species, all traits or characters are inherited through discrete "factors" (units of heredity), contributed by each parent to the offspring. However, Mendel's path breaking discovery remained unnoticed till it was rediscovered and revived by Thomas Hunt Morgan and others scientists in 1900s. Wilhelm Johannsen introduced the term "**Gene**" for the unit of heredity in 1909 and it was established that genes are arranged linearly on the chromosomes. British physician Archibald Garrod (1857 - 1936), introduced new concepts by publishing "In-born errors on metabolism " in 1909 where he explained genetic defect. Self-replication and governance of cell function are the two minimum prerequisites of hereditary material. But nobody had any idea how all these are carried out by chromosomes.

In the first half of the twentieth century, not only biologists, but many chemists and physicists were attracted to the study of genes. Max Delbruck, Salvado Luria, Alfred Hershey were a few of them. Due to the work of all these eminent scientists the science of **Genetics** evolved. In 1941, George Beadle and Edward Tatum established the theory of one gene one protein. In 1944 the famous physicist and Nobel laureate Erwin Schrodinger wrote the book "*What is Life*", where he stated genes to be the **information carriers**. He also wrote that the only reasonable way in which genes could be imagined to carry their hereditary information is by embodying a succession of a small number of different repeating elements, or symbols, whose exact pattern of succession represents an encoded genetic message.

In fact, nobody initially thought that DNA was the hereditary material. At that

time, mainly due to the work of Phoebus Aaron Theodore Levene (1834 - 1917), one of the first to propose protein as genetic material, the idea prevailed that DNA was simply an equimolar tetranucleotide structure and this led to the rejection of DNA as hereditary material for a long time. Chromosomes contained both DNA and proteins. Proteins, which appeared to be much more complex, seemed the most likely candidates as carriers of heredity. For the first four decades of the twentieth century, many scientists believed that the proteins found in the cell nucleus are the hereditary material.

The first direct demonstration that DNA is the genetic material was provided by Oswald T Avery (1877 - 1955) and his collaborators at the Rockefeller Institute in New York in 1944. Avery had shown that upon addition of purified DNA, extracted from normal donor bacteria to abnormal recipient bacteria that differ from the donor bacteria in one mutated gene, some of the recipient bacteria are transformed hereditarily into the donor type. Thus the normal donor gene must have entered the transformed recipient bacterium in the form of a donor DNA molecule. So hereditary traits could be transformed from one bacterial cell to another by purified DNA molecule.

Avery's claim that DNA was the transforming principle, the bearer of the genetic information was so radical at that time that it met with suspicion in many quarters, in spite of the fact that Avery, MacLeod and McCarty in their new classical publication of 1944, expressed their results after experimenting with all sorts of rigorous control (like digestion with DNAase, protease etc). The well-known biochemist Alfred Mirsky, a colleague of Avery at the Rockefeller Institute, was firmly convinced that Avery's DNA preparations contained protein, the true transforming principle. Avery was very much disappointed and depressed. At this time he coined his famous phrase: *"Disappointment is my daily bread, but I thrive on it"*. Avery's discovery was much discussed in different quarters and when in 1946 the Royal Society of London awarded him the very prestigious Copley Medal, he refused to go to London for the award ceremony on the pretext that because of his delicate health, he could only travel by first class, which would be far too expensive. Avery seems to have been completely indifferent to both fame and reward.

The fact remains that this gentle, quiet little man, always very neat and as discretely dressed as an old-fashioned family doctor, is one of the towering figures in biomedical research. If Watson and Crick can be considered as the father of Molecular Genetics, there can no doubt, that the grandfather was Oswald T Avery.

As time rolled on science progressed. A highly talented biochemist, born in Vienna in a well-to-do Jewish family, Erwin Chargaff (1905 - 2002), by refined biochemical analysis of DNA, showed that DNA from any source contains equal amounts of Adenine (A) and Thymine (T), that is ( $A=T$ ) as well as equal amounts of Cytosine (C) and Guanine (G), that is ( $C=G$ ) and does not consist of equimolar amount of each of the four bases. Any arbitrary order of the bases in the polynucleotide chain is possible. Chargaff published his data on base complementarity of DNA in 1950 from Columbia University. Finally in 1952, eight years after Avery, Alfred Day Hershey (1908 -1997) and his young assistant Martha Chase working on *E.coli* and its bacteriophage  $T_2$ , with radioactive technique showed that it was the DNA of the phage that enters the cell and not the protein; thus directly demonstrating that DNA is the genetic material. After this definite evidence, all genetic thoughts were focused on DNA.

Exactly at this juncture, James Dewey Watson and Francis Harry Compton Crick entered the scene at Medical Research Council Unit, at Cavendish Laboratory, Cambridge University, U.K. for the study of molecular structure of biological systems

J. D. Watson was born on April 6, 1928 in Chicago. He obtained his B.S degree in 1950, from Indiana University working under the guidance of Salvador E. Luria (1912 -1991) on "The lethal effect of X-rays on Bacterial-virus". He joined University of Copenhagen to learn biochemistry in the laboratory of Herman Kalckar, a Danish biochemist. In the spring of 1951 in a chance meeting with Maurice H.F. Wilkins at Naples, Watson came to know about the X-ray crystallographic analysis of the three-dimensional structures of large biomolecules like DNA and he got excited. To learn the necessary skill in X-ray crystallography, Watson joined John Kendrew in Cavendish Laboratory, Cambridge in the autumn of 1951. At that time Kendrew was working on the X-ray crystallographic structure of myoglobin with Sir William Lawrence Bragg\*

\*[ William Henry Bragg and Sir William Lawrence Bragg, the father and son had invented X-ray- crystallography and were awarded Nobel prize jointly in 1912. They have founded a School of Crystallographers in Britain to study molecular architecture.]

F.H.C Crick, an English physicist, was born on June 8, 1916, and studied physics at the University College of London. He had served in World War II. After the war was over, he lost his job and also his interest in Physics; instead he decided to try Biology. He joined Max Perutz in Cavendish Laboratory for his Ph.D work. At that time Max Perutz was working on the X-ray crystallographic structure of hemoglobin with Sir. W. L. Bragg.

So, Watson and Crick met each other in Cavendish Laboratory in the autumn of 1951 centering a common theme of solving the molecular structure of proteins. But both of them realised that DNA is more golden than protein and so both of them teamed up to work on the three-dimensional structure of DNA.

To find out the correct structure of DNA, Crick and Watson adopted the technique of making physical models, arranging and rearranging the chemical pieces that DNA contained. Many facts were already known: Base complementarity (Chargaff's rule) and polynucleotide nature of DNA were already established. The concept of the helical configuration in macromolecules was already there. In 1951, at California Institute of Technology  $\alpha$ -helical structure of proteins was established by Linus Pauling (1901 -1994). X-Ray crystallographic studies by Rosalind Franklin (1920-1958) and Maurice Wilkins at King's College, London, had already indicated the double helical nature of DNA. All these findings specially the X-ray diffraction photographs taken by Rosalind Franklin and Chargaff's base complementarity rule of DNA were the two main keys which helped Watson and Crick eventually to build and interpret an accurate three-dimensional structure of DNA. Earlier they were pairing like-with-like bases, but a chance luncheon meeting with Chargaff at Cambridge in May 1952 had changed their views and they paired A with T and G with C. Their scientific vision, knowledge, intelligence, speculation, superior imagination, strong common sense and intellectual ability had enabled them to postulate the correct structure of DNA double helix .

DNA-structure hunting competitors were many and they toiled much. Most significant of them were Linus Pauling who wrongly suggested triple-helix structure of DNA, Erwin Chargaff established the base complementarity of DNA but could not take the last decisive step; Maurice Wilkins had presented number of X-ray diffraction patterns of DNA; Rosalind Franklin was the first to propose the right handed double helical structure of DNA, i.e., the B form of DNA. In the same April 25, 1953 issue of Nature three articles came out; first one by J.D. Watson and F.H.C. Crick, the next by Maurice H.F. Wilkins, A.R. Stokes and H.R. Wilson and the last by Rosalind E. Franklin and R.G. Gosling. But Watson and Crick finally turned out to be the successful winners although their steps to the top were quite twisted.

On the morning of Feb, 28<sup>th</sup> 1953, Watson and Crick had figured out the structure of DNA and on the same afternoon they went to the Eagle Pub in Cambridge and announced that "*We have found out the secret of life*". The discovery of the double helical structure of DNA with complementary bases, antiparallel, polynucleotide chains by Watson and Crick had an astounding impact on the scientific community and became a roaring success. Acceptance of the double helical structure of DNA was very swift and complete. The reason was that the model was very informative and accommodated all the basic features. Also the timing was perfect - the double-helix arrived exactly at the right time. The model of DNA proposed by Watson and Crick explained the three basic characteristics of hereditary material. It not only revealed the genetic structure, but also showed how genetic information could be expressed in the form of a chemical code, explained the process of accurate self-replication of the DNA molecules and proposed how mutation can occur in genes in terms of changes in the chemical structure of DNA. Thus the model proposed by Crick and Watson clearly explained how DNA can control the synthesis of proteins and thus can govern the characteristic features and the functioning of a living being as well as heredity and evolution.

In the annals of the history of science, the story of discovery of the structure of DNA is unique in many ways. Watson and Crick, the discoverers of DNA-double helix, were not officially working on DNA. The discovery was a unique combination of choice and chance. The historic research paper of Watson and Crick was very brief, it did not cite any authorities or historical record. The paper did not have any experimental proof - but only guess work, postulations and hypothesis. Interestingly, all their hypothesis were later on proved to be true.

In 1962, Watson, Crick and M.H.F. Wilkins received the Nobel Prize in Physiology and Medicine for their discovery of the double-helical structure of DNA. It was indeed a well-deserved prize. But there are two tragic figures in this story. One is Rosalind Franklin who had taken the key X-ray diffraction photographs that provided the information Crick and Watson needed for establishing the structure of DNA. She could not receive the Nobel prize as she died of cancer four years earlier at a young age of thirty-eight and Nobel prize is not given posthumously. The other is Erwin Chargaff who had been so close to the goal but could not take the last decisive step.

So this is the saga of Golden Helix which spans over a period of more than hundred years involving endeavour, hard work, dedication and love of so many minds.

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# Application of Animal Bio-technology in Human Welfare

PROF. ARUN K RAY

**R**emarkable progress has been made in Molecular Biology during the last few decades due to development of many microtechniques. Recombinant DNA technology and techniques of gene transfer are also exploited as main components of biotechnology for development of transgenic animals for human benefit. Till date the success rate of transgenesis in animal is very low and the implementation cost is very high. Simultaneously with the technique of transgenesis alternate innovative methods are being searched for better production of commercially important animals in a farmers friendly and cost-effective way, suitable for enrichment of socio-economic condition of developing countries. Vertebrate sex steroids have been identified in biological fluid of many crustaceans and insects including commercially important prawn and silkworms. Exploitations of such phenomenon have been made to achieve better productivity of prawn and silk. With such experimental evidences a concept of "**Endocrine Biotechnology**" has emerged. Recently understanding of the Stem Cell biology is in progress with a view to combat with many of the human sufferings.

**Animal Agriculture :** As human population in our planet is continuously growing, need for parallel massive increase in production of food is posing a threat to us. According to a United Nations Report, by the year 2020 livestock sector will be the most favored agricultural Sector and application of modern biotechnology will be the only way to achieve the goal. The different types biotechnological approaches are: **(1) Artificial Insemination (AI) :** With this technique of transfer of sperms from elite males to receptive females , about 100 million cattle, 40 million pigs, 3.3 million sheeps, 0.5 million goats are produced globally, **(2) Embryotransfer (ET) :** Frozen embryos are transferred to a receptive mother with proper endocrine bed of uterus . This is costly and mostly practiced in developed countries, **(3) Ovum-Pick-Up(OPU), IVM/IVF :** OPU involves repeated ovum pick up followed by *in vitro* maturation and fertilization, **(4) Embryo/ Sperm Sexing :** Technologies for rapid embryo sexing offers facilities to preserve the embryos of required sex. Sexing of semen through flow cytometry is another way to select the sex of sperms, **(5) Cloning/Transgenesis :** Cloning may be used to multiply transgenic founder animal. The sampling of somatic tissue may assist collection and transfer of breed samples from remote areas for conservation purpose.

**Molecular Biotechnologies :** Technologies have been developed to protect

health for the livestock. DNA technologies are being used to detect animal disease, characterization of pathogens, etc. Recombinant DNA technologies provide new opportunities for development of vaccines against parasites like ticks, helminths, etc. The production of tailor-made plant product nutritionally rich for the use as animal fodder are also made through DNA technology. For selection of breed, characterization of genetic variation and conservation of genetic materials DNA technologies are also used.

**Stem Cell Biology :** The stem cells are 'blank cells' those may be transformed to any tissue in the human/animal body. They could thus be used to create a stock of spare body tissues. Stem cells are pluripotent or multipotent in nature and self-renewing. Depending on the source they have the potential to form one, many or all cell types of an organism. Stem cell could be derived from embryo, fetal source, cord blood, placenta, liver, prostate, neural, mesenchymal, endothelial, adipose tissues, etc. **The major obstacles and risks in use of stem cells(embryonic) are** — Non availability of human embryo, lack of public research funding, most of the data are based on animal experiments, human embryonic stem cells need to be experimented in primate model, method for transplanting human stem cells and monitoring for their proper development in the host environment needs to be worked out. Tumor formation and immunorejections appear to be the major risks in handling stem cells. About, at least one mutation occurs in a cell division cycle. After about 200 cell division cycles 200 mutations will be there in embryonic stem cells. It is a question to ask — are they safe for human transplantation ?

**Current Status of Adult/Embryonic Stem Cells in Medicine :** Although the topic is at present in experimental stage, evidences show formation of kidney tissue from bone marrow stem cells in humans. Similarly, hepatic and pancreatic tissues have been made. Use of adult stem cells have cured diabetes and liver failure in rats. Rat bone marrow stem cells could be differentiated *in vitro*, when transplanted they fused with host cells in rat. None of the application of embryonic stem cells have been made clinically. Embryonic stem cells have been found to be differentiated to cardiac cells, nerve cells, liver cells, insulin producing cells, *in vitro*. Risk for tumorigenesis always remains for use of the embryonic stem cells.

**Benefits of Human Genome Project :** Human genomes have very recently been screened. **Gene test**, the newest and most sophisticated techniques related to direct examination of the DNA molecule itself. **Carrier Screening, Preimplantation Genetic Diagnosis, Confirmational Diagnosis of Symptomatic Individual, Forensic/Identity Testing**, are some of the applications of human genome project.

**Gene Therapy :** The function of a faulty gene can be suppressed by inserting a correct gene or by repairing the faulty gene itself- this is the concept for 'gene therapy'. Several approaches have been thought for viz., a normal gene could be inserted to a non-specific genome to replace a non-functional gene, an abnormal gene could be swapped for a normal gene by homologous recombination, abnormal could be repaired by selective reverse mutation, etc.

**Vectors for Gene Therapy :** There are several means for carriage of the correct gene in the host cells like — use of Retroviruses which create double stranded DNA

from RNA and copies of its genome can be integrated into chromosomes of the host cells are — Adenovirus with double stranded DNA, Adeno-Associated Virus with single stranded DNA and Herpes Simplex Virus ( double-stranded DNA virus that infect a particular cell type) have also been taken into account for this purpose. The difficulty lies with the short-live nature of the gene therapy and the patient may undergo multiple treatment. Recently several developments in the gene therapy have been made. In University of California at Los Angeles, genes have been inserted into brain using PEG-coated liposome. Gene silencing (RNA-DNA hybrid) has also become a target to manage Huntingtons chorea . Several approaches to repair errors in m-RNA have been made to cure some diseases like thalassaemia, cystic fibrosis, some type of cancers. Attempts have been made to carry therapeutic DNA in 25nm liposomes and pass through the nucleopores. Sick cell anemia has been successfully treated in mice through gene therapy.

**Endocrine Biotechnology :** We have found before how knowledge of molecular biology has been applied for various technologies to transfer gene of required quality either to over express it or to correct a faulty expression. All such techniques are very costly and need sophisticated laboratory for handling. All those maneuvers appear to be out of reach for developing countries where cost-effective farmers-friendly techniques for improvement of agriculture will be well-suited for their socio-economic condition. Therefore alternative ideas have been grown to over-express a particular gene coding for a particular quality by its receptive ligands which already exist in the animal system. Endocrine biotechnology is an outcome of such a concept.

Estradiol-17 $\beta$  (E2) is a vertebrate female sex steroid and its actions are well known in the vertebrates. In oviparous vertebrates it induces the vitellogenin(egg-yolk-precursor) gene in liver in the breeding season and causes accumulation of vitellogenin in plasma which are taken up by the developing oocytes for further maturation. E2 has also been found in body fluid of some crustaceans and insects including giant prawn and silkworm. Further it has been found that E2 has tremendous metabolic potentials in both the commercially important animals. Thus exogenous E2 treatments potentiates growth and early ovarian maturation in freshwater prawn. E2 also improves some commercial parameters of silk production. Such effects are of much importance in prawn culture and sericulture. Indian catfish, singi (*H. fossilis*) is nutritionally very rich and considered to be an important menu in the diet. Fish farmers are very reluctant to culture this fish because they breed once a year in the rainy season. Endocrine approaches have been successful to break the seasonal barrier and induce the fish to breed twice a year. Use of endocrine biotechnologies are very easy, farmers friendly and cost-effective. The skill of development of such techniques depends on clear understanding of the exact moment and duration to hit the particular gene of importance by application of its natural ligands.

# Modern Chemical and Pharmacological Perspective of Herbal Medicine

PROF. BISWAPATI MUKHERJEE

Plants were worshipped and regarded as more valuable than gold not only in India but also in other parts of the world where the modern civilisation sprouted. This was mainly due to their magical power of providing relief to different ailments, associated with the creation of mankind. In the earlier days the application of plants in medicine was based on empirical observations. In the meantime the science of Chemistry, Pharmacology, Physiology, Molecular Biology, etc. progressed with tremendous speed and were applied to establish the importance of plant medicine on a firm footing. With this modern outlook and application of sophisticated chemical, biochemical and indepth pharmacological techniques we want more from the plants for fighting against the existing and incoming dreadful diseases. This article is designed to focus the modern trends of research on phytomedicine.

Let us first start with the story of an old woman of Shropshire, a county in U.K., who cured cases of congestive heart failure, discarded by the English Physician William Withering around 1775. The main constituent of the drug was the leaves of the plant *Digitalis purpurea* L. and it belongs to the family *Scrophulariaceae*. Upto this stage it was herbal medicine only. Then chemists intervened and isolated and identified a particular group of chemical known as glycosides, more specifically cardiac glycosides<sup>1</sup>. These essentially contain a steroid nucleus consisting of three cyclohexane (A,B,C) and one cyclopentane (D) rings flanked by sugar and lactone. These rings have specific steric structures and they are joined in different geometric fashions, known as conformational isomers. In the cardiac glycosides the A/B ring juncture is *cis* and B/C and C/D are *trans*. The lactone ring and CH<sub>3</sub> groups are  $\beta$ -oriented. Any change in the orientation of these substitutions will destroy the effect of the glycoside as cardiotonic agent<sup>2</sup>.

There has been tremendous advancement in the biochemistry and molecular biology in the last three decades. Cell, the basic building block of our body has been exposed to a great extent. The cell is just like a well protected factory. It is surrounded by a wall which has got well defined gates, channels, pumps, etc. for the entry and exit of different ions under immaculate control and the normal physiological functions are carried out by well synchronised orchestra of communication among trillions of cells through signalling of superb intricacy. The cardiac glycosides are working in an interesting way which is different from hormonal steroids<sup>3</sup>. Thus the efficacy of the herbal medicine is now tested in the molecular level to establish its scientific claim or to look

for new activities which were not known before.

The plant *Atropa belladonna* was used by Italian women to beautify their eyes and the crude drug was used to treat many ailments. The intervention of chemistry led to the isolation of an alkaloid, later known as *Atropine*. The structure was fully divulged. It was established that atropine was a racemic mixture of (+) and (–) hyosciamine, an alkaloid possessing a property known as *optical activity*. This normally occurs in compounds having 'asymmetric' carbon atoms i.e. carbon containing four different groups which produces two enantiomers behaving oppositely towards polarised light. This asymmetric centre is better termed as 'chiral centre'. Chirality is a unique property of naturally occurring compounds. Nature can easily synthesize millions of chiral specific compounds, i.e. a particular compound which a particular rotation (+) or (–). But it is very difficult to synthesize chiral specific compounds in the laboratory, particularly complex herbal drugs having more than one asymmetric centres. This property greatly influences the pharmacological activities of the drugs.

The most shocking example is the thalidomide disaster. Thalidomide was used as innocent drug by expectant mother to get relieved from uneasy symptoms. Unfortunately it was detected afterwards that thalidomide had teratogenic effect and produced children with limb deformities. The teratogenecity was eventually traced to the (S) - (–) - thalidomide whereas (R) - (+) - enantiomer is claimed not to cause deformities in animals even in high doses. The tragedy could be avoided had the physiological properties of the individual thalidomide enantiomers been tested prior to commercialization. This necessitated the change in the regulations of Food and Drug Administration (FDA), USA. It is now mandatory to submit the informations about the enantiomer composition of chiral substances in new drug applications. New terminology had been introduced to explain the medicinal properties of stereoisomers. The biologically more active isomer of stereoisomeric pair has been named *eutomer*, the corresponding less potent or inactive isomer is then called *distomer*<sup>4</sup>.

Now turn to opium poppy, the scientific name of which is *Papaver somniferum* L. The power of opium poppy, both for good and for evil, has been known since at least 3000 B.C., when the Sumerians called it the joy plant. By 300 B.C. opium was being used by Arabs, Greeks and Romans as a sedative and soporific. The most important of opium poppy's constituents, morphine (named after Morpheus, the Greek God of dreams) was isolated in 1803 by a 20-year-old German pharmacist named Friedrich Wilhelm Adam Serturner. It was the first plant alkaloid ever isolated and its discovery set off a fire storm of research that changed medicine forever.

On examination of different derivatives of morphine viz., heroin, codeine, levorphanol, opioid antagonists and agonists it comes out that there is a common moiety present in each structure to which the pharmacological activity of opium alkaloids may be attributed. Based on this moiety structure of the basic receptor for analgesic activities of morphine and their derivatives was visualized<sup>5</sup>. After 1970s there evolved a completely new concept in this arena. In 1975, Huges became the first of a number of independent investigators to report the presence of chemicals in brain extracts that behave like morphine on pharmacological preparations. The compounds were called ENKEPHALINS. For obvious reasons two active peptides are called

LEUCINE (or LEU) and METHIONINE (or MET) – ENKEPHALIN. Thus a natural system is present in the body that can selectively release various opiopeptides in response to pain and other stimuli. Morphine and narcotic analgesics apparently mimic the action of these endogenous ligands by binding with their receptors; this interaction gives rise to pharmacological effects<sup>6</sup>.

Herbal medicine has been very helpful in providing an excellent lead for synthesizing better drugs for the treatment of different ailments. The development of antiasthmatic drug<sup>7</sup> salmeterol, salbutamol, etc. using ephedrine, isolated from several species of the Chinese plant *Ephedra*, as lead compound and the antimalarial drug<sup>8</sup> chloroquine, mefloquine, etc. taking quinine, isolated from the *Cinchona* plants, as the basic nucleus, may be cited as examples.

Normally the plants are processed for the isolation of active principle following their tribal, folklore or ethnic use in a particular disease. But it has been observed that continuous chemical pursuance may lead to compound or compounds which may be effective against diseases not mentioned earlier. The best example is the Madagascar periwinkle *Vinca rosea* which was used in Jamaica for antidiabetic effect. But two minor alkaloids vincristine and vinblastine isolated from this plants are used to treat childhood leukaemia and Hodgkin's disease respectively which were not indicated from its ethnic use<sup>9</sup>.

Our immune system saves us from certain death by infection. Primary objective of the plant medicine as claimed by the Ayurvedic physician was to modulate immune system to prevent or cure disease by the application of plant products known as *rasayana*. The transplantation surgery now requires good immunosuppressants to prevent graft rejection. The fungal metabolites cyclosporin A, cyclosporin B and more potent FK506 are used clinically for this purpose. Intensive research is going on to develop ideal immunosuppressant drugs from plants<sup>10,11</sup>.

Free radicals, particularly oxygen species are now thought to play an important role in many types of cellular injury leading to various diseases. The generation of free radicals are aggravated during stress. So there is great demand for the anti-oxidants, free radical scavengers or antistress drugs. Extensive modern studies on some plants following Ayurvedic literature have produced many antioxidant preparations<sup>12</sup>.

Sometimes the bioassays take lead over chemistry to establish the efficacy of herbal drugs. The development of taxol as an anticancer drug is a unique example. Taxol, the active component of the extract of *Taxus brevifolia*, the Pacific yew tree, was isolated in pure form in 1969 and its structure was established by Wani, Taylor and Wall in 1971. After conventional screening taxol was not considered for the development as an anticancer drug by NCI, USA<sup>13</sup>. Interest in taxol increased in 1979 when Schiff and Horwitz described its unique mechanism of cytotoxicity<sup>14</sup>. In contrast to other antimitotic agents which inhibit the polymerisation of tubulin, Taxol promoted the assembly of tubulin and stabilised the resulting microtubules<sup>15</sup>. Ultimately taxol was established as an important drug for the treatment of ovarian cancer. This type of bioassay guided research led to the development of many potential drugs from herbal source. The development of anticancer drugs, anisodamine and anisodine, harringtonine, homoharringtonine, nitidine, camptothecin and the anti-AIDS drugs michellamine B,

calanolide A, costatolide, conocarvone, prostatin from different plants is in progress in different parts of the world<sup>16,17</sup>.

The drugs so far developed have been arrived at from chemical processing of 35,000 plants and screening of bioactivity of 25,000 extracts. This is laborious and time consuming process. To make quick assays of thousands of analogous compounds a new branch of chemical science has recently developed, that is known as *combinatorial chemistry*. The idea of combinatorial chemistry is to make a large number of chemical variants all at one time; to test them for bioactivity, binding with a target, or other desired properties; and then to isolate and identify the most promising compound for further development. Combinatorial chemistry in combination with computational drug design has opened a vast field for identifying active compounds following the basic compound either natural or synthetic<sup>18</sup>.

Molecular similarity / dissimilarity methods are emerging as important tools in drug design. Computer graphics have been applied for pinpointing the biologically active part of a chemical compound<sup>19,20</sup>.

A revolutionary technique involving molecular biology has been introduced in 1999 for designing new molecules to treat the disease in genetic level. That is known as **Microarray Analysis**. Microarray consists of ordered sets of DNA molecules of known sequence fixed to solid surfaces – detects thousands of genes in a small sample simultaneously and analyzes the expression of those genes. By comparing the ways in which genes are expressed in normal and diseased heart, for example, scientists might be able to identify the genes and hence the associated proteins – that are part of the disease process, i.e. the DRUG TARGET. This information can be used to procure drugs, natural or synthetic that would interact with these proteins, thus reducing the effect of the disease on the body<sup>21</sup>.

Another serious problem has emerged out due to over exploitation of medicinal herbs. 25% (i.e. 60,000) plant species will disappear by 2050<sup>22</sup>. Let us clarify it with an example. As discussed earlier taxol isolated from *Taxus brevifolia* is now a marketed drug for refractory ovarian cancer. 24 kg of taxol per year is required in the United States alone. Given the yield at that stage of one gram of taxol from approximately 13 kg of dried bark (equivalent to about 1.5 mature trees), the anticipated demand of over 24 kg required the processing of 3,12,000 kg from about 36,000 mature trees. The discovery of taxol's efficacy in the treatment of breast cancer created potential demands exceeding 400 kg per year in the United States. It goes beyond calculation the plant material that will be required to meet up the demand for the whole world. Naturally *Taxus* plants will be extinct species within short time<sup>17</sup>. The threat for extinction of plants for this purpose and other natural processes is also true. Chemists and Biotechnologists are trying to solve this problem. What are the alternative ways for obtaining the drugs derived from natural products without disturbing biodiversity?

The medicinal plants can be protected by synthesis of active molecule. Synthesis of giant molecules, particularly with several chiral centres are difficult, time consuming and costly, therefore, practically not feasible for commercial production. Sometimes the target molecule is obtained by semisynthetic conversions of natural precursors which are abundantly available<sup>23</sup>.

The trees can be grown for the production of active components but there are certain practical difficulties.

The other way to obtain extracts for the preparation of medicines from plants is to obtain it through micropropagation and culture of plant cell, tissue or organ. These alternative sources offer a number of benefits including uniformity of products, freedom from climatic factors, seasonal variation, disease and political restraints. To date, there is only one commercial example of plant tissue culture for the production of a higher plant natural product and that is *Lithospermum erythrorizon*, which is used to produce antiseptic dye shikonin<sup>22</sup>.

Recently genetic engineering and biotechnology has been applied to solve this problem which can be exemplified as follows. Alkaloids are important group of plant metabolites which are used for medicinal purpose. Plant alkaloid genes can be functionally expressed in microorganisms to produce either by single biotransformation steps or short biosynthetic pathways. Likewise, using over expression or antisense or consuppression technologies, medicinal plants can be tailored to produce important pharmaceutical alkaloids by introducing side pathways, eliminating side pathways, or accumulating biosynthetic intermediates. The gene for the key enzyme strictosidine synthase has been sequenced and expressed in *Escherichia coli*. Strictosidine is the precursor of thousands of indole alkaloids including vinblastine, vincristine, reserpine, etc. So plant biotechnology appears to be on the threshold of exciting discoveries, some of which may be exploited for the benefit of human beings<sup>24</sup>.

The concepts of bioavailability – enhancer and role of minor plant components in controlling bacterial Multi Drug Resistance (MDR) Protein Pump are going to revolutionize the science of herbal drugs. Traditional medicine practitioners of India had suggested that the common black pepper, taken along with certain extracts, might increase the potency of a drug. Recent work, particularly in two Indian modern biology labs, has confirmed this bioavailability-enhancer ability of pepper, and point to the active component as the molecule piperine. It has been recently shown that piperine is absorbed very fast across the intestinal barrier. It may thus act as a modulator of cell membrane dynamics and help the transport of drugs across these barriers – by forming a complex with drugs and helping them reach the target site rather than spreading out. Piperine acts as a bioenhancer of the anti-tuberculosis drug rifampicin. In the presence of piperine the amount of the drug to be taken can be reduced in dosage, so that side effects of the drug can be minimised. This indeed is the greatest benefit of a bioenhancer substance – it helps reduce the drug dosage and thus the side effects, by directing the drug to reach the target site and not be lost on the way<sup>25</sup>.

The medicinal plant *Berberis fremontii* is used in Native American traditional medicine. This plant extract is used as an antibacterial. However, bacteria wisened up to it in the course of their evolution; most of them have pumping system in their membranes, called MDR, which sense these drug molecules and the moment they enter the cell, pump them out of harm's way! In effect the bacterial MDRs render berberine and other alkaloid antimicrobials ineffective.

The plants too would not have taken it lying down, but would have adapted through evolution! Thus it is that the plant *Berberis fremontii* and a couple of others of the family

have been seen to have another minor component in them called 5' methoxy hydnocarpin, or MHC for short. It has now shown that MHC disables or inhibits the MDR pumps in microbial cells. As a consequence, the drug berberine will not be pumped out of the cell when MHC is also present. It will enter, effect its action and kill the bacterium<sup>25</sup>.

Thus the approach to the research on plant medicine has radically changed. The modern concepts of molecular pharmacology have been combined with sophisticated chemistry for the development of drugs from plant sources.

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# Interaction between Drugs and Nutrients

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**N**utrition is the science of foods and nutrients are the food substances which provide energy to the organism by the action, interaction and balance in relationship to health and disease.

Medicine and drugs are foreign compounds that help people to recover from illness or are used to illegal substances that lead to bodily harm and addiction.

Nutritional status has a profound effect upon the response of an organism on metabolism and pharmacokinetics of foreign compounds.

Nutrients, stimulants, drugs comprise the chemical microenvironment of an organism. A complex interrelationship exists between diet and homeostatic mechanism within the body.

Drugs interact adversely on nutrient intake, function and requirement by modifying appetite, impairing digestion, inhibiting absorption, inducing gastrointestinal losses, altering organ structure and function etc.

Conversely, nutritional status affects drug metabolism by increasing bile secretion, gut enzymes, enhancing intestinal motility, altering intestinal drug metabolism etc. Deficiencies of nutrients cause decrease in detoxification and hence increasing toxicity. The microsomal cytochrome  $P_{450}$  mixed function oxidase system is sensitive to nutritional deficiencies. Decreased structural and biochemical integrity cause cells to become easily damaged, cellular replacement becomes delayed, enzyme synthesis decrease and susceptibility to drugs increase.

The present day scenario in modern medicine is extremely complex. Physician prescribe medicines in speciality disciplines which may become isolated from many others interacting factors in patient care. Added complexity arises by availability of non-prescription drugs across the counter. Networking between physician, nurses, dieticians and pharmacists is essential for proper patient care.

WHO estimates that only some 150-200 drugs are actually needed to take care of almost all ordinary illnesses. However, the market is flooded with more than 50,000 drugs, many of which are slight variation of other drugs. All persons at any age are at risk to harmful drug or drug nutrient interaction. However the elderly and child population are more vulnerable.

Because -

- Drugs are probably consumed for longer period by the elderly.
- Drugs may be more toxic due to cumulative effect.
- Both elderly and children respond to drugs with greater variability.
- They are less capable to handle drugs efficiently.
- Nutritional status may be deficient.
- Illness, mental confusion in the elderly may increase error in medication.

Several possible mechanisms coexist of nutrient drug interaction in malnutrition. This is observed in hospitalised patients, elderly who reside in institutions, persons and children who suffer from protein energy malnutrition (PEM) because of -

- Decreased intestinal absorption
- Increased renal excretion
- Direct competition or displacement from carrier protein sites
- Interference with synthesis of necessary enzymes, coenzyme or carriers
- Hormonal effect on genetic system
- The drug delivery system
- Components in drug formulation

Other factors also play a role

- Time of drug intake
- Route of drug administration
- Before or after ingestion of food

Nutrients and drugs can interact in several ways-

- Drugs can alter food intake and absorption, metabolism and excretion of nutrients.
- Food and nutrients can alter the absorption, metabolism and excretion of drugs.

## EFFECT OF DRUGS

A. Many medicines can lead to malnutrition by interfering with food intake. Drugs can influence appetite, alter taste and smell, cause sores or irritation in the mouth, reduce flow of saliva or induce nausea.

- Altering the appetite (Amphetamine suppress appetite)
- Interface with taste or smell (methotrexate changes taste sensation)
- Induce nausea or vomiting (digitalis)
- Change the oral environment (phenobarbital cause dry mouth)
- Irritate GI tract (cyclophosphamide induces mucosal ulcers)
- Cause sores or inflammations of mouth (methotrexate cause painful mouth ulcer)

- B.** Altered nutrient absorptions - laxatives cause food to move rapidly through the intestine shortening the time span of passage thereby decreasing absorption of fat soluble vitamin etc. by
- Changing the acidity of the digestive tract (antacids interfere with iron absorption)
  - Altering digestive juices (cimetidine improves fat absorption)
  - Altering motility of digestive tract (laxatives increase motility causing malabsorption)
  - Inactivate enzyme system (neomycin reduce lipase activity)
  - Damage mucosal cells (chemotherapy causes damage to mucosal cells)
  - Binding to nutrients (antacids bind to phosphorus)
- C.** Altered drug absorption : interactions between simultaneous intake of tetracycline and calcium or iron causes binding of calcium / iron with tetracycline thus reducing absorption. Interaction occurs between acidic foods and nicotine by
- acting as structural analog (anticoagulant and vitamin K)
  - competing with each other for metabolic enzyme systems (phenobarbitol and folate)
  - Altering enzyme activity and contributing pharmacologically active substances (monoamine oxidase inhibitors and tyramine)
- D.** Altered drug excretion. Acidity of urine affects the reabsorption of drugs back into the blood by kidneys. Acidic urine limits excretion of aspirin by
- Altering reabsorption in the kidneys (some diuretics increase the excretion of sodium and potassium)
  - Displacing nutrients from their plasma protein carriers (aspirin displaces folate)
  - Medicines alter urinary excretion (some diuretics accelerate excretion of calcium, magnesium, zinc)

### EFFECT OF FOODS

Dietary carbohydrate and fats influence hepatic drug - metabolizing enzyme, through various mechanism such as limiting cofactors, alteration of phospholipid composition etc.

- Changing the acidity of digestive tract (candy can change the acidity thus dissolving slow acting asthma medication)
- Stimulating secretion of digestive juices (griseofulvin is absorbed better when taken with food that stimulates release of digestive enzymes)
- Altering rate of absorption (aspirin is absorbed more slowly when taken with food)
- Binding to drugs (calcium binds to tetracycline limiting drug absorption)
- Competing for absorption sites in intestine (dietary amino acids interfere with levodopa absorption)

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Foods can also alter drug excretions in various ways

Changing the acidity of the urine (vitamin C can alter urinary pH and limit excretions of aspirin)

### OTHER FACTORS

Besides the active ingredients, medicines usually contain other substances such as sugar, sorbitol, sodium. When medicines are taken regularly or in large doses, patients on special diet need to take care.

**Sugar** : Patients with diabetes need to take care of the amount of sugar present in the medicine dose.

**Sodium** : Antibiotics and antacids often contain sodium. Patients with sodium restricted diet need to take care.

**Grape Juice** : Grape fruit juice increases felodipine oral availability in humans by decreasing intestinal CYP3A protein expression.

**Antioxidant fruit and vegetables** : These have anticancer prospects.

**Smoking** : A major risk factor for cardiovascular disease to cigarette smoking which starts in early adolescence and continues in adulthood. Tobacco smoke causes a large number of complex manifestations from immunological effects to causation of cancer.

### FACTORS AFFECTING INCREASED DRUG ABSORPTION

**Dissolution characteristics** : When a drug has poor in vitro dissolution characteristics, prolonging the time it remains in the stomach with food may increase its effective dissolution and consequent absorption.

**Gastric emptying time** : Delayed emptying of food from the stomach can have an effect of doling out small portions of a drug, creating more optimal saturation rates on the absorptive sites in the small intestine.

**Nutrients** : Some nutrients promote absorption of certain drugs. For example, high-fat diets increase absorption of the antifungal drug griseofulvin. This drug is fat-soluble, and high-fat diets stimulate the secretion of bile acids, which aid in absorption of the drug. In another example, a rice diet has been shown to aid absorption of chloroquine. Also, iron absorption is enhanced by vitamin C and gastric acid.

**Blood flow** : Food intake increases splanchnic blood flow, resulting in an increased absorption.

**Nutritional status** : In addition to the presence of specific nutrients, nutritional status may also affect bioavailability of certain drugs in different ways. For example, chloramphenicol is absorbed more slowly in children with protein-energy malnutrition (PEM), but elimination of the drug is slower in well-nourished children. In both cases the effect is a net increased bioavailability of the drug. Dysplasia by oxygen treatment have been protected by vitamin E administration during the acute phase of respiratory distress requiring oxygen treatment.

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## TO SUM UP

The field of nutrient-drug interaction is in its infancy. More research is needed to sort out the complicated permutations of possible effects.

Drugs can have multiple effects on the body's absorption, metabolism, retention, and status of nutrients. Drugs can provoke adverse reactions in combination with certain foods. Among these reactions are flushing, hypoglycemia, the disulfiram effect, and the influence of monoamine oxidase inhibitors (MAOIs) on the metabolism of vasoactive amines, which can be quite serious. Antidepressant MAOIs when combined with food or drink high in tyramine, dopamine, or related amines, can lead to a hypertensive crisis. Drugs can also influence appetite, either repressing it or artificially stimulating it. Drugs can either increase an individual's absorption of nutrients, or more commonly, decrease absorption, sometimes leading to clinical deficiencies. Drugs can also induce mineral and vitamin deficiencies.

Just as drugs affect our utilization of food, food affects our utilization of drugs. Food can affect the absorption of drugs in a variety of ways: by changing the amount of time the drug stays in the stomach, by influencing the digestive system, by increasing splanchnic blood flow, or by regulating the release of drugs into the systemic circulation. Foods also have an effect on the subsequent distribution and metabolism of drugs. Certain food, notably the cruciferous vegetables (brussels sprouts, broccoli, cabbage, cauliflower,) affect hepatic metabolism via enzyme induction. A related effect is seen from charcoal-boiled foods.

**Table 1. Drugs Causing Primary Nutrient Malabsorption**

Drug	Use	Nutrients lost	Action
Cholestyramine	Holds bile acid: hypocholesterolemic agent	Fat, fat-soluble vitamins A, D, and K; vitamin B <sub>12</sub> ; iron	Binding agent for bile salts and nutrients
Colchicine	Antigout agent	Fat; vitamin B <sub>12</sub> provitamin A (Carotene); lactose; sodium, potassium	Enzyme damage; inhibits cell division; structural defect
Methyldopa	Antihypertensive agent	Vitamin B <sub>12</sub> , folic acid; iron	Unclear; possible auto-immune action
Mineral Oil	Laxative	Fat-soluble vitamins A, D and K; provitamin A (carotene)	Nutrients dissolve in oil and are lost in feces

Drug	Use	Nutrients lost	Action
Neomycin	Antibiotic	Fat; vitamin B <sub>12</sub> nitrogen; lactose sucrose; sodium potassium, iron calcium	Binds bile salts; lowers pancreatic lipase; structural defect
Para-amino salicylic acid	Antituberculosis agent	Fat; folic acid, vitamin B <sub>12</sub>	Blocks mucosal uptake of vitamin B <sub>12</sub>
Phenolphthalein	Laxative	Calcium, Potassium; Vitamin D	Rapid intestinal transit; loss of structural tissue integrity
Potassium chloride	Potassium replacement	Vitamin B <sub>12</sub>	Lowered ilcal pH
Salicylazosulfa- pyridine (Azulfidine)	Anti-inflamma- tory agent (ulcera- tive colitis)	Folic acid	Blocks mucosal uptake of folic acid

**Table 2. Drugs Causing Secondary Malabsorption of Calcium**

Drug	Use	Action
Phenytoin, phenobarbital, Primidone	Anticonvulsant agents	Accelerated vitamin D metabolism
Diphosphonates	Paget's disease (increased bone resorption and deformity)	Vitamin D hormone [ 1.25(OH) <sub>2</sub> D <sub>2</sub> ] formation decreased
Glucocorticoids, such as prednisone	Collagen disease; allergies	Calcium transport decreased
Glutethimide	Sedative	Impaired calcium transport
Methotrexate	Leukemia	Folic acid antagonist- acute deficiency of the vitamin

**Table 3. Examples of Drugs that act as Vitamin Antagonists**

Target Vitamin	Drugs
Vitamin K	Coumarin anticoagulants
Folic Acid	Methotrexate Pyrimethamine Triamterene Trimethoprim
Vitamin B <sub>6</sub>	Cycloserine Hydralazine Isoniazid Levodopa

**Table 4. Interactions Between Oral Contraceptive Agents (OCA) and Vitamins and Minerals Affecting Nutritional Status**

Nutrient affected by OCA	Effect	Clinical result
<b>Vitamins</b>		
Retinal (Vitamin A)	Impairs liver storage; increases plasma binding	Unclear
Pyridoxine (Vitamin B <sub>6</sub> )	Alters metabolism of tryptophan and vitamin B <sub>6</sub>	Abnormal protein metabolism; mood changes
Cobalamin (Vitamin B <sub>12</sub> )	Reduces vitamin B <sub>12</sub> serum levels	Unclear
Folic Acid	Reduces red cell concentration; increases folate-binding protein	Megaloblastic anemia
<b>Minerals</b>		
Copper	Increases plasma levels of ceruloplasmin	Unclear
Iron	Increases serum levels of transferrin	Unclear
Zinc	Reduces serum levels of zinc	Unclear

**Table 5. Adverse Drug Reactions Caused by Alcohol and Specific foods**

Type of reaction	Drugs	Alcohol / Foods	Effects
Flushing	Chlorpropamide (diabetes)	Alcohol	Dyspnea, headache, flushing
	Griseofulvin		
	Tetrachlorethylene		
Disulfiram reaction	Aldehyde dehydrogenase inhibitors :	Alcohol Foods containing	Abdominal and chest pain, flushing, headache, nausea and vomiting
	Disulfiram (Antabuse)	Alcohol	
	Calcium carbimide		
	Metronidazole		
	Nitrofurantoin		
	Sulfonylureas		
Hypoglycemia	Insulin-releasing agents :	Alcohol	Mental confusion, weakness, irrational behavior, Unconscious- ness
	Oral hypoglycemic drug	Sugar, sweets	
Tyramine reaction	Monoamine oxidase inhibitors (MAOIs):	Foods containing large amounts of tyramine :	Cerebrovas- cular accident (CVA), flushing, hypertension
	Antidepressants such as		
	phenelzine	Cheese	
	Procarbazine	Red wines	
	Isoniazid (isonicotinic acid hydrazide)	Chicken liver Broad beans Yeast	

**Table 6. Food Effect on Drug Absorption**

Absorption reduced by food	Absorption delayed by food
Amoxicillin	Acetaminophen
Ampicillin	Amoxicillin
Aspirin	Aspirin
Demethyl Chlortetracycline	Cephalexin
Doxycycline	Cephadrine
Isoniazid	Digoxin
Levodopa	Furosemide
Methacycline	Sulfadiazine
Oxytetracycline	Sulfamethoxine
Penicillin G, V(K)	Sulfamethoxypyridazine
Phenethicillin	Sulfanilamide
Phenobarbital	Sulfasymazine
Propantheline	Sulfisoxazole
Rifampicin	
Tetracycline	

# Drugs and Chemical Toxicity in Humans

PROF. MALAYA GUPTA

**N**o drug or chemical is absolutely safe. Most of the valuable drugs are the most dangerous. The toxicity of a newly discovered drug has to be assayed in the light of the purpose of use, period of administration and effective dose.

## **A. Functional, Biochemical and Structural Drug Toxicity**

The toxic properties of a drug can be classified into three ways —

- (a) Functional toxicity is due to pharmacological effects, which are not necessary for the desired action.
- (b) Biochemical toxicity refers to these drug-induced organ changes which are routinely detected by chemical method.
- (c) Structural toxicity indicates all drug related macroscopic and microscopic alterations of tissues and organs.

## **B. Frequency and Organ Distribution of Biochemical and Structural Toxicity in Human**

The frequency of toxic changes was highest with corticosteroids and anticancer drugs. In patients treated with female or male sex hormones, antirheumatics and anticonvulsants, the incidence was more than 10%.

Toxic manifestations involving the hemopoietic system are the most frequent (99 cases), followed by liver (78), skin (60), sensory organs (36), pericarditis (33), influence on the fetal development (34), bone & joints (16), gastrointestinal tract (13), and kidney (10). Other organs were only occasionally involved.

## **C. Classification of Drug-Induced Toxic Manifestations in Humans**

Unwanted effects of drugs are usually subdivided into six classes :

- (a) Overdose, (b) Intolerance, (c) Hypersensitivity, (d) Side effects, (e) Idiosyncrasy and (f) Secondary effects.

## **D. Establishment of Causal Relationship between Drug and Toxic Manifestations**

- (a) Identification of the compound : authentication of the medicine has to be confirmed. Errors can occur during manufacturing and dispensing of the drug and most frequently in the patients own medicine cabinet.

- (b) Proof of drug consumption : it has to be ascertained whether the drug has been taken in the proper form of prescribed doses.
- (c) Time relationship : immediate drug toxic action (anaphylactic action) or delayed action that has to be noted.
- (d) Re-exposure : one of the most direct approaches to determine the causal relationship is readministration of the drug in order to reinduce a previously observed untoward reaction.
- (e) Differential diagnosis : it has already been pointed out that newly appearing symptoms coinciding with the administration of drugs may be completely unrelated and be due to a different disease.
- (f) Skin test and serological investigations : skin eruptions account for atleast 50% of all drug-related toxic manifestations. The methods to be used are 1. Patch test, 2. Scratch test, 3. Conjunctival test, 4. Interdermal test and 5. Passive transfer test. Agglutinin titer and seroimmunological investigations are to be done.
- (g) Consideration of supplementary drug therapy : drug itself is not toxic but due to the administration of another drug a product may form which is toxic in nature.
- (h) Consideration of pharmaceutical ingredients : chemical adjuvants are normally inert. Due to the contamination it becomes toxic in nature.

## E. Design of Toxic Experiments

In any toxicity experiment, animals are treated with drugs and observed for toxic manifestations. Toxicity experiments can be classified into three types - 1. Acute toxicity, 2. Subacute toxicity and 3. Chronic toxicity.

- (a) Acute toxicity : the purpose of an acute toxicity test is to determine the nature and extent of the untoward reactions which might follow the administration single dose (or an overdose) of the drug.
- (b) Subacute toxicity : it is a vaguely defined term for an experiment in which a drug is administered for a limited period, about 2 to 6 weeks and upto 18 weeks. Observations and measurements are same as in chronic toxicity test.
- (c) Chronic toxicity : in chronic toxicity tests, the drug is given in amounts several times greater than the pharmacology active dose and frequently enough to ensure that a high plasma concentration is maintained throughout a period of testing. It is usual to divide the screened experimental animals into a number of groups that receive different doses of drug. Throughout the test, the general appearance and conditions of animals, their weights and their food intakes are noted. Regular hematological examination, organ functional test, autopsy, histopathology, reproductive activity and special tests like teratogenic, carcinogenic and mutagenic tests must be undertaken.

## F. Drug-Unrelated Factors, Affecting the Outcome of Animal Toxicity Experiments.

The drug itself is not toxic but due to other factors it becomes toxic —

- (a) Composition of diet : impaired nutrition may influence the outcome of toxicological test in many ways.
- (b) Sex : quantitative sex differences in drug metabolizing liver enzymes may induce drug toxicity in humans.
- (c) Age: young animals are often more sensitive to drugs than adults. So same dose of the drug may produce severe reactions in case of children and old people.

Drug action is unpredictable in case of children due to incomplete development of hypothalamus.

In old age, the response is altered. This may be due to

- i) Slower metabolism
  - ii) Poor renal excretion
  - iii) Better absorption
  - iv) Increased permeability of the Blood Brain Barrier
  - v) Higher sensitivity of the receptor.
- (d) Spontaneous disease : toxic manifestations may be due to contamination by other infections. The drug itself may not be toxic at all.
  - (e) Environment : most compounds are found to be more toxic at higher temperature and under stress.

Classic example is the action of penicillin and kanamycin. Penicillin produces adverse reactions at high temperature. High dose (twice daily for nine days) of kanamycin produces nephrocalcinosis in 40% rats whereas severe calcinosis in all the rats with same dose after 17 hours mobilization.

- (f) Heredity : genetic factors modify drug action.
- g. Endocrine status : any disturbance in the hormonal balance of an organism may influence action metabolism and toxicity of drugs. It is almost a rule that drug toxicity increases in hyper-thyroid animals. Thyroxine accelerates the metabolism of Zoxazolamide and shortens the paralytic action. Thyroxine decreases the activity of Hexobarbital metabolizing enzyme and potentiates the Hexobarbital hypnosis.

## CONCLUSION

Drug induced side effects have been called disease of medical progress. Since there are no active drugs without undesired side actions, no toxicological experiment will ever be able to assure complete safety for their use in humans.



# Advances in Life Science Research: A Treasure Hunt for Disease Management

DR. MANOJ K CHAKRABARTI

**L**ife sciences involve the study of living organisms, from their molecular and biochemical subsystems to the ecosystems created by the interaction of multiple species. Life science research over the last 25 years has yielded a dramatic explosion of knowledge in this field, through a combination of conceptual advances and technical breakthroughs.

Today's understanding of the continuity of life is the fact that the very fundamental elements of life — the genetic material, DNA, and its transcription products, RNA and then protein are shared by all organisms from bacteria to yeast, from plant to animals upto humans. Furthermore, living organisms do not share only common molecules but also similar mechanisms of deriving a broad range of functions from the interactions of these molecules.

Microbiology is one of the major fields of life science. Different pathogenic microorganisms are responsible for a vast number of infectious diseases of humans, plants and animals. The "seeds" of disease which was thought to be invisible, transmissible agents were first seen when Antony van Leeuwenhoek (1632-1723) made microscopes with sufficient magnification. The science of Microbiology began with the letter of Antony van Leeuwenhoek in the Philosophical Transactions of the Royal Society of London in 1677. Advances in the development of microbiology of pathogenic organisms began with the discovery of fungi, a causative agent of a disease of silk worms by Agostino Bassi in 1836 and human skin disease (favus) the association of which with fungi was first demonstrated by Sehonlein in 1839. In 1865 Pasteur entered the field of pathogenic microbiology with the discovery of a protozoon that was threatening to ruin the European silkworm industry. The etiological role of bacteria in anthrax was unequivocally established by Robert Koch in 1876, and was confirmed by Pasteur and his medical colleague, Joubert.

Diarrhoeal disease is one of the major public health problem caused by different microorganisms. The present discussion will be restricted to the application of research findings in the management of diarrhoeal diseases only. Diarrhoeal disease has long been clinically recognized, even it was mentioned in the Old Testament. In India, the Sanskrit word believed to denote cholera is visuchika, which found a place in the Sushruta Samhita, the age of which is difficult to ascertain but it is estimated to have been written about 500-400 BC or around the time of Lord Buddha. The disease was so devastating in earlier days that in 1817 an epidemic of cholera gave birth to a new deity 'Ola Bebee'

in West Bengal. Due to its acute public health problem learned people started thinking the ways to prevent diarrhoeal diseases. During early 19th century two great notions were prevailing amongst the scientists. These are: Miasma theory and Germ theory. According to Miasma theory cholera and other diseases are caused by life forms that arise spontaneously from swamps and putrid matter (also known as the Spontaneous Generation theory) and according to Germ theory diseases such as cholera are caused by the activity of microorganisms within the body, some of which are too small to be seen. John Snow in 1845 during an epidemic in the city of London was able to use careful logic and quantitative epidemiological methods to identify the germ origin of cholera, with no recognition during his lifetime of *Vibrio cholerae*, the organism that causes cholera. He also drew attention to the intestines and their discharges. In 1854 Filippo Pacini, an Italian physician and anatomist was first to discover *Vibrio cholera*. With his microscope he observed a unique bacillus in intestinal mucosa taken from cholera deaths at autopsy. His findings were ignored by the scientific community. Robert Koch, a German bacteriologist rediscovered, isolated, and first cultured *Vibrio cholerae* — the cholera-causing microbe in early 1884. Since the discovery of *Vibrio cholerae* a lot of research have been made on diarrhoeal diseases. Owing to the advancement of our knowledge in this field we find that whereas in 1849 cholera attacked St. Louis and 10% of the population died and half of the population developed acute diarrhoeal illness, in 1991, cholera attacked Peru and 1% of the population was affected but less than 1% of the affected population died.

Three aspects of research on diarrhoeal diseases are discussed here, such as

- Mechanism of pathogenesis of the disease.
- Development of suitable drugs, to block the disease at certain stage of pathogenesis.
- Development of a suitable vaccine against the disease.

In 1959 Dr. S.N. De in Calcutta developed a rabbit ileal loop model to produce cholera infection. By using this model he showed that *V. cholerae*, inspite of being a gram-negative organism secreted a toxin which produced the disease. Cholera toxin (CT) was first purified by Finkelstein *et al* in 1969.

CT has been taken to be the prototype of enterotoxin structure and function at the membrane level. CT is the prototype A-B subunit toxin (A/B ratio - 1:5), where B is the subunit (11.6 kDa) responsible for binding of the holotoxin to its receptor and A is the subunit responsible for intracellular changes in cyclic AMP levels. The A subunit consists of two components, generated by proteolysis. A<sub>1</sub> (21.8 kDa), containing ADP-ribosylating activity and A<sub>2</sub> (5.4 kDa), which links the A<sub>1</sub> and B subunit. The receptor for B subunit of CT is the GM<sub>1</sub> ganglioside, which is present in all eukaryotic cells. CT acts by ADP-ribosylation of the  $\alpha$ -subunit of the GTP binding protein Gs, which stimulates adenylate cyclase activity. Upon ADP ribosylation the intrinsic GTPase activity of the  $\alpha$ -subunit of Gs is inhibited, resulting in the constitutive activation of the adenylate cyclase and thus continued generation of cAMP. This results in slow onset of inhibition of NaCl absorption and stimulation of Cl secretion. This occurs via stimulation of a kinase with direct phosphorylation and activation of the major chloride channels, identified in intestinal epithelial cells, the cystic fibrosis transmembrane conductance

regulator (CFTR). In addition, cAMP activates an outwardly rectifying chloride channel located in the apical membrane of the intestinal epithelial cells.

Besides *Vibrio cholerae*, among other diarrhoeagenic pathogens *Escherichia coli* plays an important role. Enterotoxigenic *E. coli* secretes heat labile and heat stable toxins. The structure and mechanism of action of heat labile toxin is very much similar to cholera toxin. Heat stable toxin is a small peptide and it stimulates guanylate cyclase activity thereby cGMP level of the intestinal epithelial cell which ultimately causes diarrhoeal. It has been reported by us that besides cyclic GMP several other signal transduction molecules are involved in its mechanism of action.

For many centuries in China and Japan traditional herbal medications known as Kampo formulations are being used to treat diarrhoeal diseases such as cholera. In Daio- kanzo-to, a Kampo formulation Daio (*Rhei rhizoma*) is the most effective part. Recently a Japanese group showed that among many compounds purified from the Daio extract, rhubarb galloyl-tannin, a compound characterized by a polygallate structure was the most effective. To define the active component, gallate analogues similar to rhubarb galloyl tannin were synthesized. These gallate compounds inhibited all CT activities including ADP-ribosylation, elongation of Chinese hamster ovary cells, and importantly, fluid accumulation in ileal loops. Thus here the in depth knowledge of the mechanism of action of cholera toxin helped to understand the target of action of the drug.

Since the discovery of *Vibrio cholerae* in 1884 more than 100 vaccines have been prepared but unfortunately till date no vaccine could give long lasting protection. First parenteral heat-killed cholera vaccine was prepared by Robert Koch. After the purification and characterization of cholera toxin a highly promising vaccine, was first produced by Finkelstein *et al* in 1979. This oral vaccine was made from a strain called "Texas Star", that produces a toxin that has the B domain but not the A (biologically active) domain. However, when administered to American medical student volunteer it produced cholera in all vaccinees. Later on it was presumed that this diarrhoea might be due to the presence of some other toxins in *Vibrio cholerae*, such as, Zona Occludens Toxin (Zot) and Accesory Cholera Enterotoxin (Ace). Later on several other vaccines have been prepared by using sophisticated molecular biology techniques. In those vaccines all virulence genes were deleted from the *V. cholerae* strain but these vaccines were also of no use. In our laboratory we are successful in getting 100% protection by oral administration of heat-killed *Shigella flexneri* 2a in rabbit against shigellosis. This highly promising candidate vaccine is simple, easy to manufacture and inexpensive.

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# Evolution of Gene Dosage Compensation Mechanisms

PROF. R N CHATTERJEE

One of the consequence of sex chromosome heteromorphism is that there are differences in the amount of quality of the genetic materials in the two sexes. The heterogametic sex ( XX and ZW in female and XY and ZZ in male ) has a single dose of some genes which are present in double dose in homogametic sex. In different animal groups where precise differentiation of the sex chromosomes in the two sexes have been established, the need for dosage compensation has been followed as an obligatory consequence depending on the functional significance of the genes in the inactivated or lost segment of the Y chromosome. Thus, dosage compensation of X linked genes can be considered as an evolutionary strategy required to equalize gene expression between individuals possessing different numbers of sex chromosomes for sex determination. The phenomenon of equalization of the X linked gene products therefore, acts as a factor against the selection preference for a particular sex and restores the balance for the haplo-X in the sex against the diplo-X of the other. Therefore, it is reasonable to believe that strong selection forces favour it. Exceptions are however, evident in systems where females are heterogametic, for example, Ophidians, aviano and Lepidopterans.

To understand the molecular solution of such compensatory mechanisms, most of the investigators have so far been restricted mainly to the three animal groups - the nematods, *Drosophila* and mammals. These animal groups have the XX/XO type male heterogamety though the mode of sex differentiation is somewhat different in the three systems (Bull, 1983). So far, four different ways of achieving dosage compensation have been recorded, such as (a) enhancing the transcriptional output of the single X chromosome in males, e.g. *Drosophila*, (b) reduction in the level of expression from the two X chromosomes in XX animals e.g. *C. elegans*, (c) eliminating unwanted chromosomes in somatic cells, e.g. *Sciara* and (d) silencing of one of the two X chromosomes in female e.g. Mammals. As dosage compensation mechanisms found so far in insects, nematodes and mammals do not share a common ancestry it is generally believed that dosage compensation may have evolved apparently independently at least three evolutionary lineages ( Chatterjee, 1998 ). Yet, biochemical and genetical data support the hypothesis that fundamental programming for dosage compensation restores the genetic balance. The concept of genic balance means that the product level of sex linked genes bear the same relation to the average level of autosomal gene products in both sexes. Clearly, genes responsible for sex determination or sexual dimorphism are excluded from this requirement. However, a large number ( nearly 500 to 1000 genes ) of other X linked genes that code for 'house

keeping' and specialized functions, respond to genetic programming to compensate for two fold differences in the number between two sexes. Different organisms have evolved different mechanisms to compensate for the dosage differences of X chromosomes in the two sexes.

From an evolutionary standpoint, it is an obvious question how genetic programming for dosage compensation is related in different organisms. Various lines of evidence (Charlesworth, 1978, 1991, 1996) clearly suggest that the mechanisms of sex determination are greatly diverse. The parallel evolution of sex determination systems in different groups of animals strongly suggest that although a variety of mechanisms are used for determination of sex in different species, a relatively simple evolutionary for be have been involved in it. However, sex determination often involves the differentiation of the structure of the sex chromosomes. During evolution, structural changes in the Y chromosome is associated with stepwise reduction of the Y chromosome activity. The evolution of genetic inertness of the Y chromosome causes severe imbalance in gene dosage between sexes - a functional aneuploidy. The deleterious effects associated with X chromosome aneuploidy between two sexes produce a strong selection pressure to develop a regulatory mechanism for compensation. In consequence, compensatory mechanisms are adapted to restore the balance between autosomal and X chromosomal gene products. A comparative study of the mechanism of dosage compensation systems in different group of animals further suggests that it is the product of a complex evolutionary process. A scenario can be developed to explain the compensation system in different animals without greatly involving molecular mechanisms of the system. To date, the date suggest that a single principle of dosage compensation system is operative in all taxa, but there is no resemblance in terms of molecular biology. As natural selection is opportunistic and always utilise common mechanism in different taxa, it is considered that somatic dosage compensation and X chromosome inactivation in germ line of the heterogametic sex may have evolved as independent solutions of degeneration and / or absence of a chromosome (i.e. in case of XO male) in different animals. This may lead us to suggest that different systems of dosage compensation found today may be the refinement of different biochemical processes of X chromosome regulation in the whole animal kingdom. Although the imminent understanding of the mechanism of dosage compensation in different animal groups has yielded some insights into the evolution of dosage compensation, to make further progress more evidence is needed on comparative genetics and molecular biology of sex determination and dosage compensation systems, particularly for the sex chromosomes that have originated recently.

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# Current Trends in Vaccine Development with Reference to Leishmaniasis

DR. SYAMAL ROY

Vaccines represent the most commonly employed immunological intervention in medicine today. As true of any other creation the development of vaccines has grown from its vulnerable infancy to a more promising maturity. It has been a long journey since Jenner's first attempt of immunization which was tried successfully nearly 200 years ago. Since Jenner's successful trial the gestation period was broken after another 100 years when Louis Pasteur and his successors used attenuated and heat killed forms of microorganisms for immunization which is designated as the first generation of vaccine. But the risk of the attenuated form of organisms to mutate to a virulent form was always there. The second generation of vaccine was constituted of defined natural or recombinant components of whole organisms to overcome the shortcomings of first generation vaccine. Subsequently the first poliomyelitis vaccine was developed in 1955 and within the next 40 years the prevalence of poliomyelitis fell by 85% and WHO confidently predicts that it would be eradicated very soon. Currently over 80% of world's children are being immunized against major childhood diseases caused by bacteria, virus and other parasites and this figure is still rising. Protein-based vaccination thus became the time-tested, well-established public health measure of vaccination. Thus the major drawback of protein-based vaccination is that it could only elicit Th2 response whereas the other arm of immune system constituting the CTLs remained largely inactive. The most recently evolved third generation vaccine consists of DNA based immunization method that is ready to take over the mantle of second-generation vaccine. Injection of naked DNA expression vector carrying the cDNA for a particular antigen into animal muscle or skin can induce immune response against a transgene-encoded antigen. Genetic immunization can induce both humoral and cell-mediated immune response activating all the arms of the immune system. Moreover the so called CpG motif, 5'-(Pu)<sub>2</sub>CpG(Py)<sub>2</sub>-3', also known as immune stimulatory sequence (ISS), present in the plasmid backbone acts as an adjuvant and induces a more vigorous antibody and CTL response than an otherwise identical plasmid lacking the CpG motif. Subsequent studies have confirmed that CpG motifs can enhance immunity to genetic immunization and have also shown that they can qualitatively modify the immune response by preferentially including a type 1 helper (Th1) response. Such DNA vaccines have several advantages over protein vaccine such as ease of construction, low expenses of mass production, high temperature

stability and long lasting immune response including CTLs. DNA vaccines thus have initiated the dawn of a new era of vaccine research as they offer an extremely powerful tool to develop designer vaccines and other immunotherapeutic approaches.

Prevalence of leishmaniasis, which mainly affects the developing world is a major parasitic disease in India. Visceral leishmaniasis is life threatening if left untreated. Visceral leishmaniasis is characterized by a wide variety of immunopathological consequences such as hypercellular spleen, hepatic granuloma and defective cell mediated immunity. Currently vaccines against visceral leishmaniasis do not exist. In recent years this disease has gained clinical relevance in humans since the infection has been described opportunistic in association with AIDS. The development of an effective vaccine strategy against the disease still eludes the scientists mostly due to the absence of a major immune reactive molecule(s) needed for mounting T-cell mediated immune response by the host against *Leishmania donovani* parasites.

Recent study from our group has demonstrated that priming of BALB/c mice and hamster with UR6(MHOM/IN/1986/UR6) provided strong protection against subsequent virulent *Leishmania donovani* challenge. UR6 lacks LPG (Lipophosphoglycan) but shows abundant message for KMP-11. Identical experiment with another avirulent *Leishmania donovani*, R2D2 (lacking KMP-11 and LPG) showed significantly less protection. KMP-11 is a surface protein, which is implicated as a parasite antigen for host protection. Thus we become interested to study immunoprophylactic and immunotherapeutic potential of this molecule in the form of DNA vaccine. Scanning of cDNA sequence of KMP-11 shows that this molecule contains two prototype immunostimulatory CpG motif in its coding sequence. Priming of BALB/c mice with KMP-11 cDNA in the Blue script vector either alone or in combination with IL-12p35/p40 expression vector showed significant level of reduction in splenic parasite burden. The level of protection was much less than when IL-12p35/p40 expression vector was given alone. Maximum protection was observed when a combination of IL-12 and KMP-11 cDNA was given to BALB/c mice. To our knowledge this is the first report concerning the host protective motif, present in the antigen of leishmanial parasites in the form of DNA vaccine.

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# Dynamic DNA Elements in Plant Genomes

PROF. RAJAT CHAUDHURI

**P**lants had evolved from a single celled condition where three genomes -nuclear, mitochondria, and chloroplast interplay amongst themselves, though they have distinct genome identities. A pro-plant cell engulfed two distinct prokaryote genomes and transformed itself into a photosynthetic organism. The circular plant mitochondrial genome, unlike the animal counterpart, harbors an extensive amount of repetition in DNA sequences. Please note that within a mitochondrion there are several circular DNA molecules (genomes). So, plant mitochondrion has genome heterogeneity.

Unlike bacteria and most viruses, plant and animal nuclear (chromosomes) DNAs are linear in structure. In each chromatid, one Mb (mega base-pair) linear DNA molecule resides. In plants, the situation is further complicated wherein intra - nuclear and intra-chromosome structures are highly repetitive. Moreover, plants' genetic makeup makes them able to coordinate three organelle genomes to function and are fit to exchange their organelle genomes also.

For nuclear genome, extreme cases are noticed in many plant genomes, such as lily, broad bean, etc. where there are almost no 'unique DNA'. The logical question is - *why there is a DNA repetition in plants' nuclear and also organelle genomes ?* The answer lies in plants' adaptability to tackle the adverse force of the Mother Nature. During evolution land plants became immobile and were exposed to environmental stress like high or low temperature, submerged or desert habit, saline or alkaline or aquatic condition. So land plants cannot move during stresses or attacked by predators. So, the very survival of a land plant depends on its genetic make-up, i.e., accumulation of extra DNA copies (non-gene sequences!) or even duplication of an intact chromosome/ chromosome segments.

Since the dawn of molecular biology, in 1960s, one basic question broods the scientists: *which is the main DNA element and what is the purpose of different DNA species?* Most DNA 'species' or 'elements' are attributed to repetitive DNA sequences. But, do highly variable repeats represent gene sequences? As gene (exon)-conservation is the rule rather than an exception, and chromosome synteny (will be discussed later) is prominent in cereals, and perhaps in all plant groups, it is hypothesized that highly labile repetitive DNA elements have something to do with plant evolution/diversification. It is pertinent here to mention that evolutionary rate of repetitive

DNAs is many time more than that of single copy DNA sequences. Moreover, repetitive DNAs harbor mobile (transposon) elements. It was proved that often they control gene function (reviewed by Chaudhuri, 1975; Flavell, 1982; Chaudhuri and Chaudhuri, 1994; Valbot, 1996; Ranjekar, 1998) and thus provide better equipments for the Mother Nature to play with.

## MACRO-LEVEL (CHROMOSOME) PLANT GENOME VARIABILITY

What are the types of macro-level genome variation in plant genomes? With modern methodology the chromosomal DNA (chromosome) can be viewed at different levels. They could be categorized into three broad types:

**First type :** In many plants, there are extra chromosomes, which are known as accessory or B chromosomes. Their structures differ from those of other chromosomes (autosomes) and they have heterocyclic staining behavior. This heterocyclic behavior is the property of heterochromatin that means of repetitive DNA sequences.

B chromosomes were discovered first in an insect (*Diadbotrica* species), in 1908. From 1950's their presence was noticed in many plants. In 1955, Darlington and Wylie had reported B chromosomes in 139 plants. By thirty years, that number has crossed over a thousand plants (Jones, 1982). If one looks at their distribution a pattern comes out. B chromosomes are common in herbs and rare in woody plant species (e.g., *Ficus krishnae*, *Betula monosperma*), also they are rare in inbreeding species and common in out-breeding species. Similarly, they are rare in polyploid species but frequent in their diploid counterpart. So inclusion of B chromosomes is the exclusion of extra chromosome (genome) sets. During mitosis and meiosis, B chromosomes separate in a random fashion, and it is believed that their movement along the cell axis is independent of autosome movement via the spindle fiber.

Functions of a B chromosome are hypothesized as (a) exotypic and (b) endotypic. Among the exotypic effects prominent effects are (i) change in color in achene, (ii) leaf stripes in maize, (iii) effect on seed germination etc. Among the endotypic effects prominent ones are: (iv) effect on chiasma frequency, (v) effect on cell division, (vi) growth and development, (vii) variation in nuclear DNA amount, (viii) variation in cell protein amount, etc. Earlier it was established that polyploidy has an adaptive significance. These observations suggested that these minute chromosomes have adaptive significances. Plants can have extra protection by B chromosomes or extra (repetitive) DNAs!

**Second type :** This type of macro-level genome variability is genome duplication (polyploidy). Polyploidy is a common feature in plants. So, during somatic cell division, by an environment cue, the entire chromosome set or a part of it duplicates. If that situation finds support from the nucleus- cytoplasm balance, the duplicated chromosomes survive within the nucleus, and autopolyploids arise. However, if those extra chromosomes disrupt normal gene functions, duplicated chromosomes get eliminated, one after another and aneuploidy ensues. During natural inbreeding and out-breeding of plants often chromosome segments may be duplicated by unequal crossing over or virus or transposon actions.

In vegetative multiplying plants, a single root cell may contain such abnormal cells - a single root may show chromosome mosaicism in adjoining cells. If those cell enter in duplication phase a meristem arise, then the ensuring plantlets would show different chromosome counts. In a natural population, often, such chromosome variations are noticed in many plants. C- value paradox is a known phenomenon in plant genomes. One-way to look into this is the presence of aneuploidy/euploidy in plant genomes. That is the basis for C-value paradox, which can be recorded by DNA measurement, with the help of flow-cytophotometer or micro-spectrophotometer. Chromosome complements can be analyzed under a compound microscope.

**Third type:** A third type of macro-level genome variability is cryptic change in chromosome structure - leading to karyotype changes. This is prominent in vegetative reproducing plants but in plants that are propagated by seeds also often show such changes. Cryptic changes are intra- chromosome changes that can be viewed by modern molecular biology techniques or by chromosome banding.

### MICRO-LEVEL (DNA) GENOME VARIABILITY

With a few exceptions DNA (deoxyribonucleic acid) is the universal genetic material. It is a high molecular weight double-stranded long molecule that can be linear or circular. Its dynamic property is the basis of evolution- its diversity at 'gene' level and that is followed by Natural Selection. When one looks through this global evolution chapter he or she would find that only four bases of a DNA molecule have oriented themselves in an infinite way within many forms of DNA molecules of all organisms. The mechanism starts at bases — their modifications, sequence re-arrangements, base pair mismatch repair, and mutation. Through standard protocols one can gain enough information on such changes. The popular methodology is DNA typing ("DNA fingerprinting"), which is based on the principle that no two individuals' DNAs are identical. That means that the level of dynamic DNA molecules in this globe is so enormous that no two DNA molecules of ten (or hundred thousand?) million living organisms are identical. The question now is

(i) How that is possible?

and (ii) What is that driving force?

The eukaryote genome is considered to be composed of different DNA species — unique DNA sequences and repetitive DNAs (Britten and Khone, 1968). Within the former, message for genes reside. Since 1960's DNA sequence diversity was measured by reassociation kinetics, thermal melting profiles, Southern hybridisation, and polymerase chain reaction (PCR). One prominent feature in these protocols is the use of probe or marker. Here comes one basic question "**which DNA marker for what purpose?**"

As one moves from small to large plant genomes, essentially most DNA "species" can be attributed to repetitive DNA sequences. The absolute amount of single copy appears to remain meagre in large plant genome where genome replication is the rule rather than an exception. In maize, broad bean (*Vicia faba*), lily, and in many other plants, no more than a very small percentage of genome appears to consist of single-copy DNA sequences. It is important to note that many *kinetic measurements*, with

single-copy DNAs, are overestimates because the reassociation kinetics was performed at criteria where extensively diverged "fossil" repeats displayed single-copy kinetics. An additional complication is that extensive short-repeat sequence interspersion makes it difficult to find single-copy sequences much larger than several kbp (kilo base pairs) in all but small genomes e.g. *Arabidopsis*.

As gene conservation is a rule rather than an exception there would be little variation in gene sequences amongst related taxa. However, evolutionary rate of repetitive DNA species is many times of that of a unique DNA sequences. Moreover, repetitive DNAs often may be highly mobile (transposon). So it is difficult to comprehend why Mother Nature tolerates the extra burden if these extra DNA copies have no function! Later it was noticed that many genes have many copies. They could be pseudogenes or alleles. But most repeats often they control gene function (reviewed by Chaudhuri and Chaudhuri, 1994). By doing so repetitive DNAs provide tools to the Mother Nature to play with. And, to measure biological diversity the best bet would be to look into gene-control control elements' sequence-diversity.

### Microsatellite instability

Microsatellite instability is one among these manifestations of genomic instability. It corresponds to an alteration in size of simple repeat sequences like simple di- or tri-nucleotide repeat motifs. A finding of microsatellite instability implies the presence of mutations in at least one gene involved in DNA mismatch repair mechanisms. Thus, though a method to detect microsatellite length constitutes an important tool in genetic characterisation of cultivars, it does not allow one to detect major forms of genomic instability such as molecular aneuploidy arising from deletions, amplifications, translocations, insertions, recombination and chemical alteration.

In plant tissue culture somaclones develop. Chromosome instability is a feature of somaclones. ISSR technique should be useful to detect major types of somaclonal variation using oligonucleotide primers like (CAA)<sub>5</sub>, (CAG)<sub>5</sub>, (GACA)<sub>4</sub> and (GATA)<sub>4</sub>. DNA was prepared from cauliflower leaves or calli cultured *in vitro*. Calli alterations were detected as gains, losses in the pattern of amplified bands. Testing of 38 calli on agarose gels revealed polymorphic markers for three calli named CH2, CJ2 and CM4. The polymorphism within calli was obvious presenting distinct fingerprints from each of them with clear polymorphic markers.

### Influence of palindromic motifs

Occasionally one would notice in a eukaryote genome an inverted repeat sequence, a palindromic-inverted repeat. Since single-stranded DNA cannot bend enough, adjacent nucleotides on one strand can create hydrogen bond like base pairs. There is a mandatory 3-6 bp spacer or loop between the 5' and 3' stems that allow the DNA to fold back on it. These stem-loop structures lead to the formation of hairpins (single-stranded DNA) or cruciform (both strands of the duplex DNA). Regions of DNA alternative secondary structure pose a barrier to replication fidelity. Previous studies in *E. coli* have demonstrated that the extruded cruciform facilitates frame shift mutations by bringing the DNA slippage sites (direct repeats) into close proximity. This can lead to deletion as well as insertion mutations.

Theoretically, like perfect palindromes imperfect ones also can mediate similar mutations and, in addition, be involved in intra- and inter-strand switching, which increases the spectrum of potential mutation. Intra-strand switching requires that the polymerase replicates through the centre of the palindrome and, with the nascent chain, disassociates from the template strand to engage itself in intra-strand annealing into a hairpin structure and continue to polymerize. Inverted repeats have several biological roles, pose a special impediment to DNA replication fidelity and are associated with several human disease-related genes.

## A FEW EXAMPLES

Since mid 1980s, large numbers of data are available about the divergence of chloroplast encoded genes (RubiscoL), chloroplast ribosomal proteins, and chloroplast ATPase genes. The molecular markers could identify species, natural populations, and cultivated forms of real ginseng, many crop plants, populations of wild irises and trees like larchwood, oak etc.

SSR markers for plant genome analyses were used for the first time in 1991. Therein, Akkaya et al (1992) was the first to use its successful application in soybean genomes. Since then the progress was tremendous. At present molecular maps of many crop plants like rice, tomato, potato, sugarcane, barley, and Arabidopsis are available. It was noticed therein that microsatellite loci differ from RFLP homeoloci. Ma and Lapitan (1998), and many other workers could successfully use AFLP markers in plant genetics, e.g. in rice, barley, wheat etc. Later, use many plant molecular biologists workers reported diversity in different plant species using PCR technology.

## The situation in grass genomes

RFLP and PCR analyses generated probes that could identify the concerted genome evolution in plant kingdom. Such situation is noticed in mammalian genomes also. In those genomes, many non-gene sequences got amplified during evolution. But in different taxa often they change their sites of residence. Classical examples are bread wheat and maize genomes. In the former, with involvement of three separate genomes a few segments of chromosomes get duplicated. They also change their positions onto chromosomes. This has given rise to Synteny Concept. Later, sequence divergence in maize transposable elements (Ac and Ds) showed that some of them (e.g. Ds1 family) underwent an expansion in numbers during a restricted period of time in the genome of the maize progenitor and *Tripsacum*. Such situation was also noticed in other plant genomes where transposon activity is prevalent. Therefore, 'selfish DNA' like transposons controls the concerted evolution in plant genomics. The inverted or direct repeats, at the ends of a transposable element, start a duplication event within the host genome.

## The situation in flax genome

It is now recognized that in most plant genomes C-value paradox exists. Instability in chromosome or DNA segments was elaborately studied in flax plant. It is the concept where nuclear DNA content of a plant may vary if that grows in different climates. It was reported that when a plant moves along a latitude or longitude gradient

its genome reacts with the environment and gets amplified. Ecotypes of many plants show this phenomenon. Even in vegetative-multiplying plant species this situation prevails. Works of Prof. Sharma at the Calcutta University and D'Amato in Italy demonstrated this where adjacent cells within a single root may have different chromosome numbers. When these cells enter into germplasm line they develop into different ecotypes or ultimately into different species.

### **The situation in Solanaceae**

This plant family was studied extensively as many vegetables belong to this family and it is highly amicable for plant biotechnology researches. Members of this crop plant family show high degree of genome variation. Classical examples are capsicum, eggplant, and *Solanum nigrum*. In late thirties, Prof. P. N. Bhaduri noticed this in *Sinigrum* species complex. We supported this with DNA data.

### **The situation in Brassica**

Microsatellite instability is one of the manifestations of *Brassica* genome. Reiter *et al.* (1992) applied RAPD technology to generate linkage maps in *Arabidopsis*. Since then molecular data had emerged from plant-pathogen interaction. Specificity between host and pathogen is determined by a clear gene-for-gene interaction. For example, polymorphic combinations of probe and endonucleases could pin point pathogen loci in genetic map of lettuce and the linkage map of lettuce mildew could be established (Michlemore *et al.*, 1989). Other studies also disclosed high level of polymorphism between different genomic DNA clones, on agarose gels with ethidium bromide staining. However, refined techniques revealed a higher polymorphism on polyacrylamide gel with silver staining. It is an observed fact that detection of amplified products after PCR is enhanced on polyacrylamide gels. Results showed that variant 'GATC clones' are responsible for most instability in *Brassica* genomes.

### **The situation in *Panax ginseng***

Besides Russian ginseng, Chinese ginseng, American ginseng, and Indian ginseng are also found. They belong to different species. Russia's Maritime Province is the only place on earth where real ginseng (*Panax ginseng*) is preserved in natural habitats. An examination of the extent of genetic variability of natural ginseng populations by DNA typing revealed genome instability. An examination of the extent of genetic variability of natural populations should be the theoretical foundation for developing a scientifically based program for preserving ginseng. The molecular markers could identify species, natural populations and cultivated forms of real ginseng, and also continental and insular populations of irises and larchwood populations.

### **The situation in woody plants**

The RAPD technology has been introduced to genetic mapping of woody plants, belonging to gymnosperms (conifers and cycads) and angiosperms (Carlson *et al.*, 1991; Bucci and Menozzi, 1993; Koller *et al.*, 1993; Dunemann, 1994; Weeden *et al.*, 1994). The author noted high level of DNA polymorphism in monotypic genus *Ricinus* (castor plant *R. communis*), perhaps due to transposon action. Heterozygosity is

generally high in Douglas fir and spruce. RFLP results also support RAPD findings in woody plants. The populations showed difference in genetic variability, which often differed not only in the presence of polymorphous loci in the DNA of several plants, but in varied intensity of homologous fragments in DNA amplification profiles.

### **The situation in *wild iris***

Today, there is no valid classification for *Iris*. Using RAPD technology a few authors typified the DNA representatives of Russian far eastern irises. Each of the *Iris* species has its specific spectrum of RAPD products characterized by a specific set of amplified DNA fragments.

### **The situation in *ferns***

Ferns are primitive groups of plants. But their genome complex is enormous—ranging from a few chromosomes to thousand chromosomes (in case of *Ophioglossum*). Surprisingly, those thousand chromosomes (definitely arisen through polyploidy) had stabilized in such a way that during meiosis no multivalent formation is noticed in *Ophioglossum*. Continued out-breeding of the offsprings with their parents, through several generations in nature, has made that situation possible. This perhaps is the situation for many out-breeding plant species.

### **The situation in *somaclones***

Somaclones are cell lines that are derived from calli or regenerated plants after performing plant tissue culture protocols. Due to the presence of growth hormones and high nutrient concentrations in the culture media chromosome instability is an observed phenomenon in plant tissue culture. As stated in earlier portion such instability in chromosome complement of a vegetative multiplying plant species was also reported in Indian labs and also from abroad. Therein chromosome mosaicism is prominent in adjacent cells of the same root-tip or meristematic bud. ISSR technique could be useful to detect major types of somaclonal variation by using tri- or tetra-nucleotide repeats like (CAA)<sub>6</sub>, (CAG)<sub>5</sub>, (GACA)<sub>4</sub>, and (GATA)<sub>4</sub>. These primers would detect micro-satellite instability or chromosome breaks, e.g. in DNA prepared from cauliflower leaves or calli (from *in vitro* cell cultures). These alterations are manifested in gain or loss of amplified DNA bands.

## **INSTABILITY IN GENE EXPRESSION AND TRANSCRIPTOME**

The intriguing observations by Spellman and Rubin pose a number of challenges about how chromosomal domains are created and maintained, why the genome contains such large clusters of similarly regulated genes, and the nature of transcriptional control. "It raises a lot of questions," says microarray aficionado Brian Oliver (NIH, Bethesda), referring to the Spellman and Rubin paper as a "call to exploration" and predicting a flood of papers exploring these domains.

Strand *et al* (1993) examined the genetic control of the stability of poly (GT) tracts and showed that the instability of poly (GT) tracts in yeast is increased by mutations in the mismatch repair genes. Thus, instability of SSRs may result from either an increased rate of DNA polymerase slippage or a decreased efficiency of mismatch pair.

In the good old days (before genome sequences and chips) the detection of quantitative changes in the expression of an individual gene (usually by Northern Blot analysis) was followed by a systematic and laborious characterization of its promoter and nearby enhancer sequences that act as a switch to determine whether a gene is on or off. This has led to models of transcriptional regulation by a precise network of sequence motifs. A predominant gene within each chromosomal territory is strongly expressed or repressed by nearby chromosome domain that usually harbors repeat DNA motifs. As long as their expression is not harmful to the cell, the changes in transcription of most genes may not be too important.

### PROTOCOLS TO NOTE MICRO-LEVEL PLANT GENOME VARIABILITY

What are the protocols to record these variations? As mentioned above, protocols evolved with time. To my personal opinion, each protocol has some advantages and disadvantages.

To monitor a DNA, refraction spectra studies including X-ray diffraction patterns of a DNA molecule can give specific clues about the base stacking and base modification. In 1960's to 1970's, popular methodologies (the classical ways) are DNA "thermal melting profiles" and DNA "reassociation kinetics studies". However, reassociation kinetics is laborious, but it could be precise, and this technique is again reviving in 21<sup>st</sup> century.

Later, with the advent of recombinant-DNA technology, after the discovery of Cohen's restriction enzyme digestion and cloning, DNA-DNA hybridization could visualize the properties of the chromosomal DNA. RFLP (restriction fragment length polymorphism) (Botstein *et al.*, 1980) is the fruit of these techniques. The application of RFLP as molecular markers has proven to be a powerful tool for studies in both basic and applied plant genetics and also to study genome evolution. The principal difficulty with RFLP is its reliance on cloning (to produce marker), Southern blotting and Southern hybridization. RFLP analysis is one of the first techniques widely used to detect variation at the sequence level. However, use of restriction enzymes and DNA-DNA hybridization, for RFLP studies, is costly and labor intensive.

Almost simultaneously, base sequence arrangement study, by DNA sequencing, by Sanger, could resolve a few properties of plant DNAs. At present sequencing has made automated. Sequencing is reliable but either it (manual sequencing) is time-consuming or it (automated sequencing) is very costly.

## DNA Xeroxing by PCR Amplification

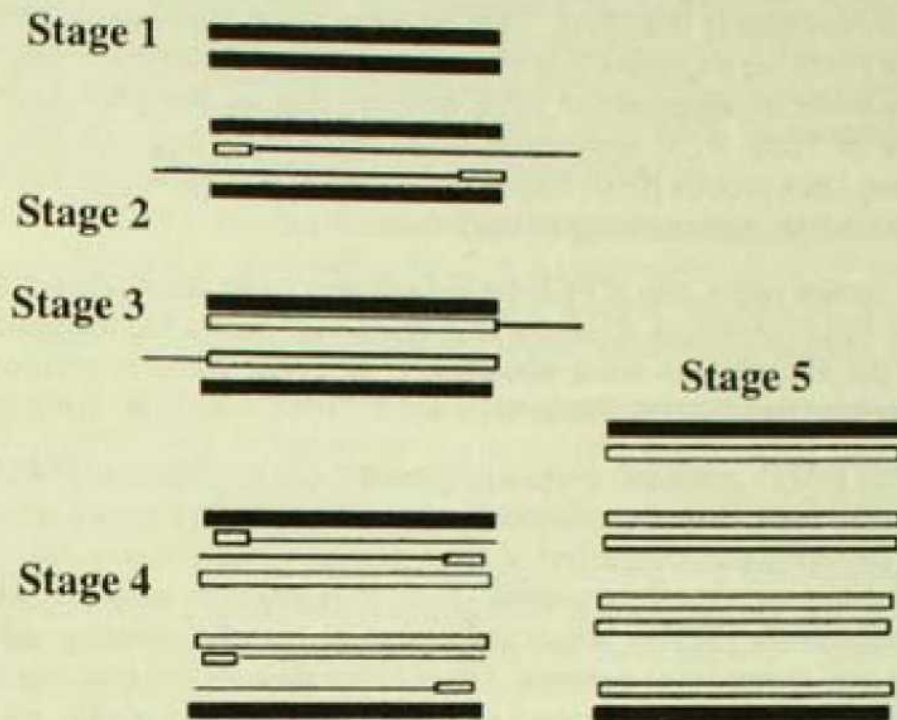


Figure 1. PCR experiments when one DNA molecule is amplified @  $2^n$  times during  $n^{\text{th}}$  replication

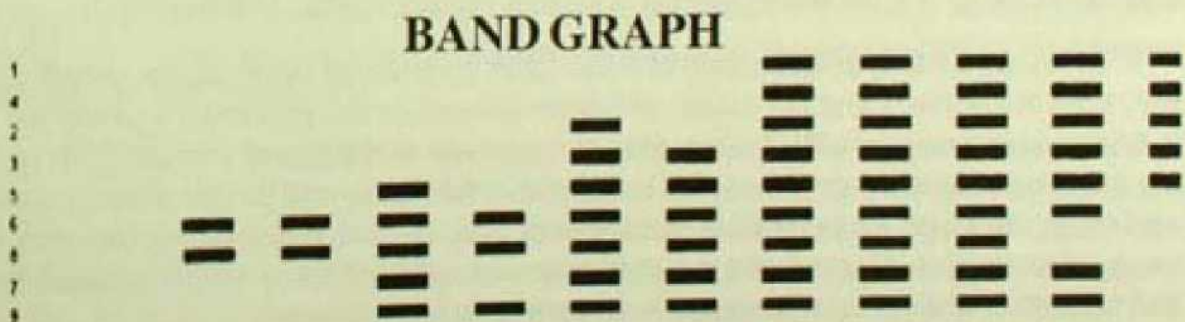


Figure 2. Bandgraph of PCR products that were generated from nine germplasms with a primer

In 1985, Dr. Kary Mullis and his colleagues developed a unique DNA replication protocol that is known as **polymerase chain reaction (PCR)** (Mullis and Falcon, 1989). This protocol can be recognised as 'Xeroxing of a DNA molecule' by repeated DNA polymerisation reactions. This protocol (now popular as '**peoples choice reaction**') is of so important that the reputed journal *Science* considered this as the major scientific development of 1989, and had chosen *Taq DNA polymerase* as the molecule of 1989. PCR techniques could replace the requirement of cloning for multiplying DNA probes (DNA fragments, marker). The applicability of this technique could earn Mullis a Nobel Prize in 1993.

In recent years, use of PCR-based markers could solve some of the limitations of earlier RFLP protocols. Over the last years PCR based techniques for identifying genetic polymorphisms were developed. Amongst them is **random amplified polymorphic DNA (RAPD)**, Williams *et al*, 1990) deserves special mention.

After RAPD, arbitrarily primed polymerase chain reaction (**AP-PCR**, Welsh and McClelland, 1990), DNA amplification fingerprinting (**DAF**), multiple arbitrary amplicon profiling (MAAP), etc. developed. Almost simultaneously it was demonstrated that microsatellites, or simple sequence di- or tri-nucleotide repeats can form simple sequence repeats (SSRs), which are useful as genetic markers, for comparative analysis and mapping of genome. Often these repeats are isolated from genomic library. However, the whole procedure, from construction of a genomic library to the synthesis of specific primers from the flanking sequences, is time-consuming and expensive. Then, Zietkiewicz *et al*. (1994) described a variant of the SSRs technique wherein microsatellite oligonucleotides that amplify genomic segments are different from the repeat region itself. This approach, named Inter-SSR (ISSR), employs oligonucleotides based on a simple sequence repeat anchored or not at their 5'- or 3'-end by two to four arbitrarily chosen nucleotides. This triggers site-specific annealing and initiates PCR amplification of genomic segments, which are flanked by inversely orientated and closely spaced repeat sequences. The study reported here was based on an inter-SSR PCR method to illustrate somaclonal variation leading to genomic instability. **The Multiple Arbitrary Amplicon Profiling (MAAP)** was suggested to describe all the characteristics common to these closely related techniques.

In **Inverse PCR**, amplification of those DNA sequences takes place, which are away from the primers and not those, which are flanked by the primers. For instance if the border sequences of a DNA segment are not known and those of vector are known, then the sequence to be amplified may be cloned in the vector and border sequences of vector may be used as primers in such a way that the polymerisation proceeds in inverse direction i.e., towards the inserted segment, and not away from it towards the DNA sequence of the vector from which primers have been derived.

**Anchored PCR** uses one specific primer that represent the precise sequences of one of the two ends of the DNA fragment that has to be amplified, because one has the knowledge about the sequence at only one of the two ends of the target DNA sequence. Therefore anchored PCR utilizes only one primer instead of two primers. By this technique, only one strand will be copied first; after that a poly G will be attached at the end of the newly synthesized strand. This newly synthesized strand with poly G tail

at its 3' end will then become template for the daughter strand synthesis utilizing an anchor primer which has a poly C sequence linked to it.

**Sequence Tagged Sites (STS) and Sequence Tagged Microsatellite Sites (STMSs)** can amplify DNA fragments of interest provided the primers are designed on specific DNA sequences (obtainable after DNA sequencing). Primers are designed on flanking sequences of the DNA (or gene) of interest. Therefore, by repeated DNA amplification intervening sequences of a DNA stretch is amplified.

A more reproducible method is the **amplified fragment length polymorphism (AFLP)**, Vos *et al.*, 1995) was developed. AFLP is a culmination of RFLP and PCR where DNAs are digested with one six-cutter and another four-cutter restriction enzymes and primers are designed on these two restriction enzyme site sequences.

Recently, with the advent of PCR technology, the task has been easier. A new addition to PCR is **DNA-chip technique** that is developing fast enough because the technique is very fast. It employs RT-PCR followed by DNA imprinting over a glass slide or a silicon chip by inject and photolithography technologies. However data generated by DNA chips need to be tallied by other techniques. During mid 1990s, DNA chips with microarrays of DNA samples became available to achieve very fast speeds in generating information about DNA sequences (reviewed by Gupta *et al.*, 1999). This is considered a major technological breakthrough in the field of DNA monitoring protocols, and that can be compared with the role of semiconductors in the field of electronics.

The microarrays are prepared on solid glass (silica) plates where high-density single stranded 20-mers DNA probes (often cDNAs) are layered. These microarrays are hybridized with an unknown labeled DNA sample, and the hybridization patterns are analyzed with computer device. With this protocol, a single experiment can reveal several thousand DNA-DNA hybridization reactions.

### Old Wine In a New Bottle

A major obstacle in genome sequencing, particularly in plants, is separating the protein- encoding genes from the repeats. Researchers at University of Georgia, Athens (UGA), using sorghum as a test case, have streamlined this process using a technique popular during the 1960s and 1970s, i.e. DNA renaturation kinetics of DNA, or popularly known as Cot curves. The revived approach is called as **CBCS (Cot-based cloning and sequencing)**.

CBCS has something for everyone, for it shatters and sorts genome pieces into three classes — highly repetitive, moderately repetitive, and single- or low-copy sequences. The technique can reveal genome size and complexity, and CBCS can greatly reduce the number of clones needed to sequence a genome by excluding repeats. BCS is the brainchild of Daniel Peterson, research coordinator with the Plant Genome Mapping Lab and assistant professor of plant and soil sciences at Mississippi State University. "It can reduce the cost of sequencing entire genomes by 50% to 95%," (statement by Andrew Paterson, Director, UGA Plant Genome Mapping Laboratory). For example, in onion, which is 98% repeats, CBCS cuts the number of required clones from 119 million to 15 million. At Georgia, Peterson and associates Alexander Nagel b cloned the three classes of sorghum DNA. "At this point, the whole Cot cloning project was just an intellectual and technical exercise, a side project."

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# Dynamic Lung Function Study of Smoker, Non-Smoker and Persons with Respiratory Diseases

PROF. SATIPATI CHATTERJEE

Over the last twenty years there have been alarming increases in the death rates from various pulmonary diseases. The increased incidence of chronic obstructive pulmonary diseases has stimulated the interest in pulmonary function testing. During the past generation a large number of physiologic tests have been developed for the qualitative and quantitative evaluation of pulmonary functions. These are now as important as are the tests of hepatic, renal, cardiovascular and neuromuscular functions, developed earlier. Tests of the pulmonary function are of the utmost utility, and play a vital role both in the diagnosis and in guiding therapy of subjects having abnormalities in the cardiorespiratory system. They have led to a better understanding of pulmonary physiology in healthy men and women of all age groups. They may be helpful for studying large population groups for comparison namely in industry or in people exposed to different environments. Measurements of pulmonary functions are also of great importance in the early detection of pulmonary dysfunction in some persons considered to be normal on the basis of clinical and radiological examination (Chatterjee *et.al.* 1987, 1988 and 1991).

Chronic bronchitis is defined as a disorder characterised by hypersecretion of bronchial mucus, usually accompanied by chronic cough or recurrent productive cough for a minimum of three months in a year for at least two successive years, in patients in whom other causes for these symptoms have been excluded (American Thoracic Society, 1961). It is one of the chronic obstructive lung diseases. Chronic obstructive lung disease is associated with abnormalities in pulmonary ventilation, perfusion, diffusion and with nonuniformity of ventilation and perfusion through the lung (Burrows *et al.*, 1965). These disturbances in lung function are reflected in the result of a variety of pulmonary function tests.

The subjects were judged to be healthy on the following criteria :

- No history, current or past, of any cardiopulmonary disorders;
- No evidence of cardiopulmonary disease from physical examination; chest roentgenogram and electrocardiogram;
- No obvious signs of weakness or debility which significantly limits activity;
- Capable of adequate co-operation during the tests.

Dynamic lung function studies included forced vital capacity (FVC), forced expiratory volume in 1 sec ( $FEV_1$ ), forced expiratory volume in 1 sec as the percentage of forced vital capacity ( $FEV_{1\%}$ ), forced expiratory time (FET), forced expiratory time of last 0.51 (FET 0.51), maximum voluntary ventilation (MVV), maximal expiratory flow rate ( $FEF_{200-1200}$ ), maximal mid-expiratory flow rate ( $FEF_{25-75\%}$ ), end-expiratory flow rate ( $FEF_{75-85\%}$ ), peak expiratory flow rate (PEFR) and flow of last 0.51.

### COMMON COMPOSITION OF TOBACCO (CIGARETTE)

- NICOTINE
- CARBON MONOXIDE
- HYDROGEN CYANIDE (Highly carcinogenic)
- BENZO PYRENE (Highly carcinogenic)
- POLYCYCLIC AROMATIC HYDROCARBON (Highly carcinogenic)
- N-NITROSO COMPOUNDS (Carcinogenic)
- N-NITROSO NOR NICOTINE (Carcinogenic)
- PHENOLS (Carcinogenic)
- TAR (Carcinogenic)

(A) The normal 'non-smoker' group consisted of subjects who denied any cigarette consumption during their life time or who had only smoked occasionally or smoked less than 5 cigarettes per day.

(B) The normal 'smoker' group consisted of subjects who had a continuous history of smoking and smoked a minimum of 5 cigarettes daily for minimum 5 years.

Most subjects were light smokers "15 cigarettes / day" and moderate smokers "15-20 cigarettes / day" and heavy smokers "more than 20 cigarettes / day".

The risk of lung cancer is greater in smokers (i) who start early in life, (ii) who take more puff of each cigarette (iii) who keep the cigarette in the mouth between puffs and (iv) who re-light half-smoked cigarettes.

In conclusion we may say that the dynamic lung functions in chronic bronchitis patients would definitely be deteriorated. And we may also infer that higher the smoking rate and higher the age, the higher is the prevalence of chronic bronchitis and lung cancer.

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# Recent Advancement in the Maintenance of Body Profile

PROF. A. M. CHANDRA

**B**ody profile now-a-days plays an important role in the maintenance of health and fitness.

Although **body profile deals** with the **muscular and non-muscular components** of the body with the help of **anthropometric measurements and computations**, here the discussion will be restricted to mainly the **body composition**.

## WHY KNOWLEDGE OF BODY COMPOSITION IS ESSENTIAL ?

Body composition means the three structural components of the human body - muscle, bone and fat.

In general terms, we usually describe human body on the basis of visual appraisal as small, medium and large or thin (ectomorphy), muscular (mesomorphy) and fatty (endomorphy).

Concept of body composition is dealing with health and fitness / rather on life span was first favoured by **Metropolitan Insurance Company** by simple considering height/ weight table. This height/weight table is one of the most common method used to make judgment about whether or not a person is overweight. **Dividing a person's weight by the mid -point average weight, a Relative Weight (RW) or Obesity Index (OI) is obtained.**  $RW = 1$  as normal,  $RW=1.1$  classified as overweight and  $RW = 1.2$  generally considered as obese.

Another measure of body composition that has been universally adapted is the **Body Mass Index (BMI)**, which is the ratio of body weight in Kg/height in sq meter. BMI index normal 19- 25 for the age group of 19-35 years, 21-27 for the age older than 35 years.  $BMI = 27.8$  obese (men),  $27.3$  obese (women.)

A height / weight table and the body mass index can indicate "overweight" relative to an average weight but **do not provide quantitative information about the composition of that weight in terms of fat free man and fat man.**

## WHY KNOWLEDGE TO BE ESSENTIAL ?

For quantification of **major structural components of the body** a wide variety of methods are now available but all are not accessible to a broad base practioners in the health and fitness professions.

Two general procedures are used to evaluate body composition (1) **Direct**  
(2) **Indirect**.

Indirect methods are mostly used in laboratories and **field studies**. As **underwater weighing**, although a good way to obtain a measurement of body density, is time consuming and requires special equipment and personal.

With the advancement of technology used in body composition analysis, scientists developed equations that **predicted body density** from a collection of **skin fold thickness measurement**. Two such equations are put forward by **Jackson & Pollock** for men and **Jackson, Pollock and Ward** for women.

Generalized equation for **men (Jackson & Pollock)**

$$1.112502 - 0.0013125 (X_1) + 0.0000055 (X_1)^2 - 0.0002440 (X_2)$$

$X_1$  = Sum of chest, triceps and subscapular skinfold

$X_2$  = Age in years

Generalized equation for **women (Jackson, Pollock & Ward)**

$$1.089733 - 0.0009245 (X_1) + 0.0000025 (X_1)^2 - 0.0000979 (X_2)$$

$X_1$  = Sum of triceps, suprailliac and abdominal skinfold

$X_2$  = Age in years

The body density obtained is used in the **Dr. William Siri's** equation to calculate % of body fat

$$\% \text{ body fat} = (495 / D) - 450$$

If we know the % fat composition of the body, we can calculate fat mass.

The body density obtained is then used in the **Siri equation** to calculate the % of body fat. There are several other formulae other than Siri equation, which have also been devised to estimate body fat from density. The basic differences in calculating body fat between the formulae are generally 1% within a range of body fat of 4 – 36%.

$$\text{Fat mass} = (\% \text{ fat} / 100) \times \text{body weight}$$

Once we are able to determine fat mass we can calculate the Free fat mass (FFM) or Lean body mass (LBM)

$$\text{FFM (LBM)} = \text{Body weight} - \text{Fat mass}$$

## HOW MUCH FAT WE NEED ?

**Classification as per Lohman is as follows**

(i) For Men

10 – 20%

above 20%

above 20 – 25%

as an **optimal** range for **health and fitness**

risk of diabetes, heart disease and hypertension

considered moderately high

25 – 31%	high
Greater than 31%	very high or obese
6 – 10%	low
Less than 6%	very low

(ii) **Females** are 3% fatter than **males** prior to **puberty** and 11% after **puberty**

15 – 25%	optimal range of body fat
25 – 30%	moderately high
30 – 35%	high
Greater than 35%	very high or obese
12 – 15%	low
Less than 12%	very low

**Exaggerated fear of getting fat = eating disorder– anorexia nervosa.**

**Desirable body weight or body mass :**

On the basis of the % fat content one can set one's body weight ranges which is desirable

**(FFM(LBM)/1) – optimal fat %**

Recent information revealed that not only the distribution of fatness is responsible for higher risk of CVD and sudden death, individuals with a large waist circumference have a higher risk of CVD compared to hip circumference. Ratio of waist to hip circumference **greater than 0.95 for men and greater than 0.8 for women** are associated with CVD, risk factors of Insulin resistance, high Cholesterol and hypertension. **Obesity** is a **real problem** for the maintenance health and fitness.

**Characteristics of obesity :**

**Obesity is of two types :- a.Hypertrophic** (increase in amount of fat in fat cells)

**b.Hyperplastic** (increase in number of fat cells)

**Moderate obesity** - adipose tissue mass less than 30kg increase in fat cell size appears to be the primary means of storing additional fat.

**High obesity** - adipose mass greater than 30kg where fat cell numbers as well as fat cell size increases.

**Dietary restriction** - size of fat cell decreases but not the cell numbers. High fat cell numbers are believed to be related to the difficulty of the obese patient in maintaining BW once it has been lost.

Obesity is related to both **genetic** and **environmental** variables

**25% of fat mass** is tied to **genetic factors** and **30%** is due to **cultural transmission**.

## WHY THERE IS A WEIGHT GAIN ?

Weight gain occurs when there is a chronic increase in calorie intake compared to energy expenditure. A net gain of about **3500 Kcal** is needed to add **454 gms** or **1 lb** of **adipose tissue**.

### Energy Balance

#### *Static equation*

Change in energy store (CES) = EI - EE

EI = energy intake

EE = energy expenditure

#### *Dynamic equation*

Rate of change of energy stores ES = rate of change of EI – rate of change of EE

ES = energy store

EI = Energy intake

EE = Energy expenditure

Dynamic energy balance equation correctly expresses the dynamic nature of changes in EI and body weight. An increase in EI leads to an increase in body weight, in turn, EE increase to eventually match the higher EI. Body weight is now stable at a new and higher value.

Nutritional balance is not a problem for protein and carbohydrate. The daily protein intake is used to maintain existing tissue protein, hormones and enzymes. If more is taken in than is needed, the extra is oxidized for metabolic needs and fat mass is not increased. The same is true for carbohydrate. Ingested carbohydrate are used to fill liver and muscle glycogen stores, excess is oxidized and is not converted to fat. Carbohydrate intake promotes its own oxidation. This is relatively new idea that has major ramifications for our understanding of nutrient and energy balance. Evidence seems to be quite convincing that de novo lipogenesis from carbohydrate is of only minor consequence in human. Simply carbohydrates are either stored as CHO or oxidized, they do not add directly to adipose tissue mass. This leaves fat.

In contrast to carbohydrate and protein, fat intake is not automatically balanced by fat oxidation. When extra fat is added to the diet, the same amount of carbohydrate, fat and protein are oxidized as before, the extra fat is stored in adipose tissue. Fat intake does not promote its own oxidation. Fat oxidation is determined primarily by the difference between total energy expenditure and the amount of energy ingested in the form of carbohydrate and protein. Consequently, if one wishes to keep the size of adipose tissue stores constant (i.e. maintained body weight) then one should not eat more fat than one can oxidize. Alcohol intake is balanced by its own oxidation, but in the process it suppresses the fat oxidation. In this sense, the calorie from alcohol should be included with that provided by fat.

The concept of the Respiratory Quotient ( $RQ = V_{CO_2} / V_{O_2}$ ) has taken up as an

indicator of the fuel oxidized during exercise. A RQ of 1.0 indicates 100% of the energy is derived from carbohydrate and a RQ of 0.7 indicates 100% fat oxidation. A RQ of 0.85 indicates 50% / 50% mixture of carbohydrate and fat.

This RQ concept has been extended to the foods we ingest and it is called Food Quotient (FQ). FQ is defined as the ratio of  $\text{CO}_2$  produced to the  $\text{O}_2$  consumed during the oxidation of a representative sample of diet. The reason for describing this is that the FQ concept can be used with RQ concept to determine if an individual is in nutrient balance.

When $\text{RQ} = \text{FQ}$ ,	the person is in nutrient and energy balance ( <b>RQ / FQ ratio is 1.0</b> )
When $\text{RQ} > \text{FQ}$ ,	the person is not oxidizing as fat as was consumed ( <b>positive energy balance</b> ) and some fat has been stored in adipose tissue, <b>RQ / FQ ratio is greater than 1.0.</b>
When $\text{RQ} < \text{FQ}$ ,	the person used more fat than was consumed ( <b>negative energy balance</b> ) and some of the fat stores were used, the <b>RQ/FQ ratio is less than 1.0.</b>

The concept is helpful in discussing weight control because one could improve nutrient and energy balance with regard to fat by either reducing the amount of fat in the diet (increasing FQ) or doing exercise to use more fat (decreasing RQ).

- Nutrient balance exist for both protein and carbohydrate. Excess intake is oxidized and is not converted to fat.
- Excess fat intake does not drive its own oxidation; the excess is stored in adipose tissues. Achieving fat balance is an important part of weight control.
- The ratio of the Food Quotient (FQ) to the Respiratory Quotient (RQ) provides good information about the degree to which an individual is in nutrient balance.

A good diet provides the necessary nutrients and calories to provide for tissue growth and regeneration and to meet the daily energy requirements of work and play. In our society we are fortunate enough to have wide variety of foods to meet these needs. However we tend to consume more than the recommended amount of fat which is believed to be related to our country's obesity problems. The focus on dietary fat is two folds:

- Fat grams contain more than twice the number of calories as carbohydrate and can contribute to a positive energy balance and
- It is difficult to achieve nutrient balance for fat when it represents a large fraction of calorie intake.

## WHY SHOULD DIET COMPOSITION MATTER IN TERMS OF WEIGHT CONTROL AND OBESITY ?

Quite simply, diet with a high fat to carbohydrate ratio are associated with obesity. When subjects are given free access to food more calories are consumed when one eats a high fat diet than when one eats a high carbohydrate diet. The high carbohydrate content may contribute to satiety better than the high fat diet, resulting in an earlier termination of eating. The nutrient balance ( $RQ = FQ$ ) concept helps focus attention on the need for a high carbohydrate / low fat diet to achieve and maintain a healthy level of body fatness. This diet is also consistent with what is needed to have normal cholesterol level and sufficient carbohydrate for physical performance.

Further to lose weight the following suggestions were made.

- Eat a variety of food that are low in calories and high in nutrients.
- Eat more fruits, vegetables and breads and cereals – without fats or sugars added in preparation and at the table.
- Eat less fat and fatty food.
- Eat less sugar and sweets.
- Drink little or no alcoholic beverages.
- Be more physically active to increase energy expenditure.

Components of energy expenditure that are influenced by genetic factors (a) RMR/BMR (b) Thermic effect of food (c) Relative rate of carbohydrate and fat oxidation and (d) the amount of spontaneous physical activity.

**RMR / BMR** is important in the energy balance.

### **Thermic and Physical activity**

Physiological set point - Booth's suggested that there is a biological set point for body weight much like the set point of any negative feed back biological control system. While the hypothalamus contains centers associated with satiety and feeding behaviour, we must remember that the body weight set point is a concept rather than reality.

Physiological model of a body weight set point in which biological signals with regard to blood glucose (glucostatic signal), lipid stores (lipostatic signal) or weight on feet (ponderostatic signal) provide input to the hypothalamus, if the collective signals indicate low energy stores - food intake is stimulated until the source of signal is diminished and energy stores now equal to the set point. If the set point were increased / to be increased, body weight would increase to meet this new value. Exercise can modify the signals going to hypothalamus and the type of diet can also influence feeding behaviour.

In contrast, to the physiological model Booth's cognitive set point deals with the role of the environment (culture, socioeconomic class etc.) has on the body weight. It

shows that relative to a personally selected "ideal" body weight set point, we are constantly receiving a variety of cognitive signals about how we look, body weight, clothings, size, perception of effort and concept about health. A mismatch between the "ideal" set point and these perceptions leads to inappropriate eating behaviour. Exercise can modify the signal and the type of diet can influence the feeding behaviour. The set point model is closely related to the behaviour modification approach to diet, exercise and weight control.

### **WAY TO REMAIN HEALTHY AND FIT**

- **Eat to live**
- **Don't live to eat**
- **Keep yourself physically active**
- **Perform exercise regularly**

# Iodine Deficiency Disorder

DR. AMAR K CHANDRA

## **E**NVIRONMENTAL IODINE DEFICIENCY AND ITS IMPORTANCE

**The Need of Iodine :** Iodine is an essential element for normal growth and development in animals and men. It occurs in the human body in only small amounts (15-20mg) and the essential requirement for normal growth is only 100-150 µg per day. Because of this iodine is referred as 'trace element'. The special biological importance of iodine arises from the fact that it is a constituent of thyroid hormones thyroxine ( $T_4$ ) and triiodo-thyronine ( $T_3$ ).

The loss of iodine from the soil due to glaciation, snow, high rainfall and flood leads to a low iodine content of all food grown in it. Inadequate dietary iodine leads to reduced synthesis of thyroid hormones –  $T_4$  and  $T_3$ . The lower level of  $T_4$  in the blood stimulates pituitary gland to secrete TSH. TSH increases the rate of pumping iodine by the thyroid from the blood and the production of thyroid hormones. There is also cell hyperplasia with the enlargement of thyroid gland that is called goiter. Persistence of iodine deficiency leads to a greater depression of the level of  $T_4$ , with a number of important consequences.

**The Consequences of Iodine Deficiency :** When the people do not have enough iodine, they cannot make enough thyroid hormone. This deficiency of iodine has several important health hazards that together are called "iodine deficiency disorders" or IDD. These are :

- Goiter
- Hypothyroidism
- Cretinism
- Reproductive failure
- Childhood mortality
- Socioeconomic retardation

**Geographical Distribution of Iodine Deficiency :** Iodine occurs in fairly constant amounts in ocean water but is distributed very unevenly in the world's crust. Inland regions far from the ocean have the greatest risk of iodine deficiency. Some of the most severe iodine deficiency occurs in mountainous areas such as in the Alps, Andes and Himalayas, where iodine in the soil has been washed away by the rain and glaciers. However, iodine deficiency is not confined to high mountains, and also occurs in large parts of Central Africa, Central Asia and Europe. It has also been associated with areas exposed to frequent flooding and in large river deltas, such as those of the Ganges, Yellow River and Rhine.

**Magnitude of IDD :** Although the quantity of iodine required by an individual does not exceed a tea spoonful for life time, its deficiency constitutes a major health problem. The estimated level prevalence of IDD at global level extends to thousand millions, of which, nearly 220 millions suffer from goiter, six millions constitute cretins and 20 millions suffer from neurological disorders.

In India, over 167 millions run the risk of IDD while 54 millions have goiter, 2.2 millions end up as cretins and 6.6 millions develop neurological disorders. There is not a single state in India which does not have iodine deficiency disorders.

### DETECTION OF IODINE DEFICIENCY

**Background :** Whether iodine deficiency exists in a particular region or population and if so, how severe it is — is the first question to ask. Usually some previous information will be available. For example, travellers or local health workers may have noted that many people from a certain area may have visible goiter. Iodine deficiency in a given region may be predicted from knowledge of its geographical location.

The most valuable means for detection of iodine deficiency in a given are

- the prevalence of goiter
- the urinary iodine excretion.

**Goiter Survey :** Goiter is usually the most obvious sign of iodine deficiency, but brain damage, mental retardation, miscarriages, and child mortality are more serious consequences. It is, therefore, important to determine the goiter prevalence in a population to determine whether these more serious consequences are likely to be present. In almost all areas, goiter occurring in a large fraction (more than 10% ) of the population will result from iodine deficiency rather than some other cause.

**Sample size and selection of Population :** It is recommended that school children be selected for assessing IDD prevalence. Children of 8 – 14 years are the ideal target group to be studied. To permit comparisons between clusters or regions at least 75 children from each of the 30 clusters in the targeted age group independent of sex have to be studied for thyroid size, i.e., a total of 3,000 children in the State.

**Classification of Goiter Size :** The very first decision should be whether or not the subject has a goiter of each lobe of thyroid is smaller than the part of the subject's thumb beyond the last joint, the thyroid is classified as Grade 0, no goiter. If each lobe is larger than the terminal phalynx of the subject's thumb, he or she has goiter. The goiters are classified in following groups

Grade	Description
0	No palpable or visible goitre
1	A mass in the neck that is consistent with an enlarged thyroid that is palpable but not visible when the neck is in the normal position. It moves upward in the neck as the subject swallows. Nodular alteration(s) can occur even when the thyroid is not visibly enlarged.
2	A swelling in the neck that is visible when the neck is in a normal position and is consistent with an enlarged thyroid when the neck is palpated.

**Urinary Iodine :** Urinary iodine reflects the iodine consumption of the individual and thus widely used as marker of iodine deficiency. In each study area at least 40 urine samples will be collected at random covering the entire age group from target population for the assay of urinary iodine.

**Other Clinical and Laboratory Data :** The goiter surveys and urinary iodine estimation are the simplest and most valuable means of assessing iodine deficiency in a population. Occasionally further laboratory investigations are obtained for research purposes or other in conjunction with other evaluations, e.g.,

- Tests related to thyroid hormones
- Neonatal screening
- Radioiodine uptake
- Ultrasonography

These tests can be valuable but they are expensive and difficult to obtain and are not usually necessary in a general evaluation.

**Severity of IDD Endemia :** Data from the goiter prevalence surveys and urinary iodine determination can be used to assess the severity of the IDD and the urgency of its correction.

IDD severity and the need for correction			
Stage	Goiter Prevalence (%)	Mean Urinary Iodine ( $\mu\text{g} - \text{l/dl}$ )	Need for Correction
Mild	10 – 19.9	5 – 9.9	Important
Moderate	20 – 29.9	2 – 4.9	Urgent Severe
Severe	30	<2	Critical

### IDD CONTROL PROGRAMME

IDD Control Programmes are the basic functional units for the prevention and control of IDD. Once the presence of iodine deficiency is established, a programme to deal with it must be developed. A model has been developed as follow :-

- **Assessment**                      Assessment of the population or group living in an area that is suspected of being iodine deficient.
- **Communication**                Transmit the message of the effects of iodine deficiency to various target groups that make up a community, e.g., Health Professionals, Politicians, General Public.
- **Planning**                          Developing or updating a plan of action.
- **Political Decision**                Achieving political will and obtaining support.
- **Implementation**                Once the political decision is made on a national IDD control programme with appropriate allocation of resources, implementation can proceed according to the plan that has been submitted.
- **Monitoring and Evaluation**    Essential for iodization programme to ensure quantitative correction of iodine deficiency.

The process then beings a further cycle with new date, dis-semination of the results of the first programme, and development of a new modified one to correct the deficiencies of the first.

## METHODS OF IODINE SUPPLEMENTATION

Once it is established that iodine supplementation is necessary, then we must have to decide the best way to provide it. The available methods for introducing iodine into a deficient area given below :

**Salt :** It is an ideal vehicle for addition of a micronutrient such as iodine. Everyone needs salt, usually in fairly constant daily amount. The sources of salt are usually limited, making them susceptible to control for addition of iodine. The added iodine does not affect the appearance or taste of salt and is usually well accepted by the consumer.

Two chemical forms of iodine, KI and  $KIO_3$  are commonly used for salt iodization. KI is cheaper but less stable. Iodate rather than iodide must be used when the salt is exposed to excessive heat and humidity, or when storage and transportation cause long delays before consumption.

**Oil :** A well known chemical reaction can add iodine to vegetable oils. The most widely used preparation, Lipiodol, is 38% iodine by weight, so 1 ml contains 480 mg of iodine. A single intramuscular injection of 0.5 – 1 ml protects from iodine deficiency for 3 to 5 years.

**Iodized Water :** Iodine added directly to drinking water can correct iodine deficiency. In the simplest form of this approach, a measured amount of iodine, usually as a concentrated solution of  $I_2$ , KI or  $KIO_3$ , is added directly to drinking water in a jar, in an amount appropriate for achieving a daily intake of at least  $150 \mu g$  iodine.

**Lugol's Iodine :** Subjects can receive oral iodine directly in the form of Lugol's solution. Lugol's solution is commonly found even in small rural hospitals in developing countries.

Iodization of salt is the preferred approach for supplementation in environmental iodine deficient populations. Salt is a dietary necessity whose sources are usually limited and therefore, easily controlled and the technology for iodization is simple.

## ENVIRONMENTAL GOITROGENS OTHER THAN IODINE DEFICIENCY

The role of iodine deficiency as the principal environmental determinant in the development of endemic goiter and related diseases is firmly established. Iodine supplementation drastically decreases the incidence of goiter, and eradicates endemic cretinism and associated neurological and developmental abnormalities. However, there is epidemiological and experimental evidence that exposure to other naturally occurring antithyroid agents magnifies the severity of the goiter endemia and may also affect the clinical expression of the associated disorders. Thus iodine supplementation does not always result the complete eradication of goiter and there have been some well-defined instances of clusters or geographical "pockets" where the condition remains endemic. There are environmental agents, which acting through the food and/or water exposure pathways, may be responsible for the exaggeration of the persistence of goiter.

Environmental compounds may cause goiter directly by acting on the thyroid gland, but also indirectly by altering its regulatory mechanism and/or the peripheral metabolism and excretion thyroid hormone. Each category of environmental agents known to have goitrogenic/antithyroid effects in humans and other animal species are given below :

### Environmental agents producing goitrogenic and / or antithyroid effects

Environmental Compounds	Source	Goitrogenic / Antithyroid Effects
<b>Sulfurated Organics</b>		
Thiocyanate	Cyanogenic glycosides (cruciferae)	IT
Isothiocyanate and its by-product thiourea	Cyanogenic glycosides (cruciferae)	IT OOBC
Goitrin	Thioglycosides (Brassica seeds)	OOBC
Disulfides	Onion and garlic water contaminants	OOBC
Flavonoids and its derivatives phenolic acids, fluoroglycinol and gallic acid	In plant as glycoside polymers	OOBC Peripheral conversion of $T_4$ to $T_3$ . Interact with TSH to inhibit its action on thyroid cells.
Phenolics, resorcinol	Water pollutant	OOBC
Pyridines	Tropical legume. Aqueous-effluents from coal conversion process.	OOBC
Phthalates Esters and Metabolites	Water pollutants, plants	IT, OOBC
Polycyclic Aromatic Hydrocarbons	Water pollutants	Accelerate $T_4$ metabolism and lowers serum $T_4$ . Stimulation of thyrotropin- thyroid axis.

Environmental Compounds	Source	Goitrogenic/ Antithyroid Effects
<b>Inorganics</b>		
Excess iodine	2 mg or more/day	P, R
Lithium	Drinking water used as medicine in manic patients	R

IT — Iodide transport; OBC - Oxidation, Organic Binding and Coupling  
P - Proteolysis, R - Release

Several categories of naturally- occurring and man-made antithyroid agents may enter the water, air and food exposure pathway, becoming an important goitrogenic environmental factor in man and other animal species. Their effects may be additive to those of iodine deficiency, making the intensity of IDD manifestation more severe. Their presence should be considered particularly in areas where the features of IDD persist despite adequate iodine prophylaxis.

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# Neurodegeneration and Neuroprotection : Epilepsy and Parkinsonism

PROF. DEBJANI GUHA

**T**he brain occupying the key position of the Central Nervous System is affected by various diseases at young and old age. Epilepsy is encountered more among the younger generation and Parkinson's and Alzheimer disease is observed to be an old age disease. Epilepsy, one of the commonest disorders of the CNS has been with us for centuries. But still today it is a mystery. The common terminology of epilepsy is - 'seizure discharge.'

## EPILEPSY

Epilepsy is a major neurological problem all over the world. Incidence and prevalence studies of epilepsy have been reported from many countries but comparisons are often difficult because investigators have adopted different definitions of epilepsy, classification schemes, and selection bias (Shorvon, 1990). Nevertheless, most studies have found incidence rates of 20-70 cases per 100,000 per year (range 11-134 cases per 100,000 per year) and point prevalence rate of 0.4 to 0.8% (Chadwick, 1990).

Incidence varies with age. The incidence rate is highest in children under 2 years of age and in persons over 65 years of age. About 30% of patients with seizures have an identifiable neurological disorder and the remainder has idiopathic epilepsy (Elwes *et al*, 1985). Males are more likely to have a new diagnosis of epilepsy than females. The seizure type and the cause of the seizure changes with age.

Epilepsy is hyperactivity of a group of nerve cells and is characterized by physical manifestation of behaviour.

The brain cells (neurons) like many other cells in the body have the built-in capacity to generate potentials or electric signals. It is through these signals that the message is transmitted to a far off part of the body. A brain cell may be receiving several messages while sending out different messages at the same time. It is normally done in a very meticulous, orderly fashion. Therefore, different functional signals or messages are not jumbled up. The brain acts like a telephone switch-board. If the incoming and outgoing calls are plugged in wrongly or there is something else wrong with the switch-board, then there is a chaos and impairment of communication. All functional activities are represented in the brain not at the same place but in different areas and at different depths. Depending upon which brain cells are impaired will be reflected in the disturbed or altered functions of that area of the brain. Malfunction of the switch-board can happen only once or frequently at different intervals and for different

durations. Epilepsy is an electric disturbance resulting from a disorderly discharge of brain cells. The discharge is sudden and recurrent in nature. The discharge may or may not be accompanied by alteration in consciousness; or motor, sensory, psychological and behavioural functions. The clinical manifestations would depend on the nature of the discharge. In epilepsy, as a disease, the discharge has to be recurrent. A single convulsion should not be taken as equivalent to epilepsy. Motor manifestations of a seizure are called convulsions. A single episode is called a seizure. The other common names for seizures are blackouts, attacks, and fits. Thus epilepsy is the disease of the brain (Benardo and Pedley, 1984).

For many years epilepsy was not considered a brain disease. Hippocrates suggested that the seat of the disease is in the brain which overflows with phlegm that clogs the veins and causes convulsions. Many people consider that epileptic seizures are caused by supernatural power while many others blame evil spirits, devils, demons or black magic and so on. Some people also regard epilepsy a mental illness and a person suffering from epileptic fits is considered insane (Maheswari, 1993).

Since epilepsy is not a homogeneous entity various factors influence prognosis. Patients with a good prognosis are those with seizures precipitated by alcohol, drugs, or metabolic disturbance (Shorvon, 1984). Patients with the poorest prognosis are those with evidence of diffuse cerebral disorder (often with intellectual or behavior disturbance); early onset seizures; partial or mixed seizure types; progressive neurological disorders; or severe epileptic syndromes (e.g. Lennox Gastaut syndrome, West syndrome). The length of active epilepsy is also important — the longer the seizures continue after the onset of treatment the worse the ultimate prognosis (Elwes *et al* 1984).

Seizure activity is characterized by paroxysmal discharges occurring synchronously in a large population of cortical neurons. This is characterized in the EEG as sharp wave or spike. The physiology of a seizure is traceable to an unstable cell membrane or its surrounding, supportive cells (Meldrum, 1990). An abnormality of potassium conductance, a defect in the voltage-sensitive calcium channels, or a deficiency in the membrane ATPases linked to ion transport may result in neuronal membrane instability and a seizure (Benardo and Pedley, 1984). Neurotransmitters (e.g. acetylcholine (Ach), norepinephrine (NE), histamine (HA) and corticotrophin-releasing factor) enhance the excitability and propagation of neuronal activity, whereas GABA and dopamine inhibit neuronal activity and propagation. Normal neuronal activity also depends on an adequate supply of glucose, oxygen, sodium, potassium, calcium, and amino acids. Systemic pH is also a factor in precipitating seizures. There may be primary defects in the GABAergic inhibitory system or in the sensitivity or arrangement of the receptors involved in excitatory neurotransmission that result in a seizure (Benardo and Pedley, 1984).

Dendritic degeneration is a non-specific finding, but may be associated with membrane changes, including receptor hypersensitivity, that could contribute to epileptogenesis. Another common finding associated with both generalized seizures and complex partial seizures is an abnormality of cortical maturation often called microdysgenesis. This may manifest as clusters of abnormally large neurons in the

cortex, or as dystrophic groups of neurons in the sub cortical white matter. It has been proposed that such abnormalities predispose to diverse types of epilepsy including primary generalized epilepsy, West's syndrome, and temporal lobe epilepsy (Ellenberg, 1986). However, their relevance remains uncertain.

Anything that disrupts the normal homeostasis of neuronal cell and disturbs its stability may trigger seizures. The most clearly established of these factors are severe head trauma, infections of the central nervous system (CNS), and stroke, although many other factors are also important antecedents. A hereditary predisposition to seizures has been suggested. Epilepsy frequently occurs in families. The parents, siblings, and offspring of a person with epilepsy are more likely than the general population to have epilepsy. This familial aggregation does not necessarily imply a genetic mechanism. In addition to genes, families share environmental exposures that may also increase the risk of epilepsy. Primary generalized epilepsies have a strong genetic contribution (Anderson *et al*, 1982; Meierkord, 1989). Patients with mental retardation and cerebral palsy are at increased risk for seizures. The more profound the degree of mental retardation as measured by IQ, the greater the incidence of epilepsy (Sundaram, 1989). The causes of seizures in the elderly are cerebrovascular disease, tumor, head trauma, metabolic disorder, and CNS infections (Scheuer and Pedley, 1990). Hyperventilation may precipitate absence seizures. Sleep, sleep deprivation, sensory stimuli and emotional stress may initiate seizures. Hormonal changes occurring around the time of menses, puberty, or pregnancy have been associated with the onset of, or an increase in, seizure activity. Other precipitating factors include fever, lack of food, and drugs. Also, antiepileptic drugs (AEDs) in excessive concentrations may cause seizures. Table 1 summarizes the common causes of seizures.

**Table 1. Common causes of seizures**

<b>Mechanical</b> <ul style="list-style-type: none"> <li>• Head trauma</li> <li>• Birth injury</li> <li>• Neoplasm</li> <li>• Vascular abnormalities</li> </ul>	<b>Metabolic disturbances</b> <ul style="list-style-type: none"> <li>• Electrolytes</li> <li>• Water</li> <li>• Glucose</li> <li>• Amino acids</li> <li>• Lipids</li> <li>• pH</li> </ul>
<b>Sudden withdrawal of CNS Drugs</b> <ul style="list-style-type: none"> <li>• Alcohol</li> <li>• Street drugs</li> <li>• Antipsychotics</li> <li>• Antidepressants</li> <li>• Antiepileptic drugs</li> </ul>	<b>Toxins</b> <ul style="list-style-type: none"> <li>• Fever</li> <li>• Infection</li> </ul>
<b>Heredity</b>	<b>Idiopathic</b>

## Classification

The International League Against Epilepsy has developed a classification system (Table 2) that combines clinical description with EEG findings. Over 90% of seizure patients may be classified using this system. Using the international classification scheme, seizures may be divided into partial, generalized, or unclassified.

**Table 2. Classification of epileptic seizure (Chadwick et al, 1989)**

Traditional classification	New nomenclature
Focal motor; Jancksonian seizure	<b>1. Partial seizures</b> (seizures begin locally) <ul style="list-style-type: none"> <li>(1) Simple (without impairment of consciousness)               <ul style="list-style-type: none"> <li>◆ With motor symptoms</li> <li>◆ With special sensory or somatosensory symptoms</li> <li>◆ With autonomic symptoms</li> <li>◆ With psychic symptoms</li> </ul> </li> </ul>
Temporal lobe or psychomotor seizures	<ul style="list-style-type: none"> <li>(2) Complex (with impairment of consciousness)               <ul style="list-style-type: none"> <li>◆ Simple partial onset followed by impairment of consciousness — with or without automatisms</li> <li>◆ Impaired consciousness at onset—with or without automatisms</li> </ul> </li> <li>(3) Secondarily generalized (partial onset evolving to generalized tonic-clonic seizures)</li> </ul>
Petit mal	<b>II. Generalized seizures</b> (bilaterally symmetrical and without local onset) <ul style="list-style-type: none"> <li>(1) Absence</li> </ul>
Minor motor	(2) Myoclonic
Limited grand mal	(3) Clonic (4) Tonic
Grand mal	(5) Tonic-clonic
Drop attacks	(6) Atonic (7) Infantile spasms
	<b>III. Unclassified Seizures</b> <b>IV. Status Epilepticus</b> (prolonged partial or generalized seizures without recovery between attacks)

## On Neurotransmitters : Involvement in Basic Mechanisms of the Epilepsies

Evidences are accumulating to support a relationship between central nervous system transmitters and convulsions (Schlichter *et al*, 1986; Purkayastha and Guha 2003). Considerable emphasis has been given in studying the cerebral metabolism of the biogenic amines in view of the possibility that affective neuronal and psychiatric disorders may be associated with abnormalities in their metabolism. Currently a great deal of interest is centred on the possible involvement of certain amino acids in the mechanism of epilepsy. This is partly the result of an awakening in neurophysiology to the paradox that a range of simple and ubiquitous amino acids which are involved in a wide range of metabolic pathways in the CNS are likely also to function as major synaptic transmitters in the brain and spinal cord.

It has been hypothesized that there is an inverse relation between seizure predisposition and levels of noradrenergic activity in brain (Jobe *et al*, 1994). Brain stem seizures (tonic and clonus extensor convulsions) are characterized by innate noradrenergic deficits and from selective lesioning of noradrenergic neurons and/or pathways (Mishra *et al*, 1994).

The increase or decrease of norepinephrine level with epilepsy is area specific. An increase in seizure severity is always associated with marked depletion of NE in the midbrain excluding the inferior colliculus (Wang *et al*, 1994).

Interestingly norepinephrine (NE) has been proposed to have both pro and anti convulsant properties (Rutecki, 1995). On the other hand dopamine (DA) has an antiepileptic action. It inhibits most hippocampal neurons. The traditional anti-convulsant action of DA was attributed to D-2 receptor stimulation in the forebrain, while the advent of selective D-1 agonist with proconvulsant properties revealed that DA could also lower the seizure threshold from the mid brain (Benardo and Pedley, 1985; Starr, 1996). The inhibitory effects of DA are derived from induction or enhancement of a calcium activated  $K^+$  conductance (Benardo and Pedley, 1985).

Cavalheiro *et al* (1994) reported that after pilocarpine administration in rats, hippocampal NE level was decreased whereas dopamine (DA) content increased. Utilization rate measurement of monoamines showed increased NE consumption and decreased DA consumption. Considerable body of evidence has indicated that the noradrenergic system provides the forebrain with substantial protection against the development of seizure activity.

Low levels of dopamine in lumbar CSF in epileptics (Hiramatsu *et al*, 1987) and in epileptic foci in human brain have been observed (Mori *et al*, 1987). Again in focal epileptic cases it has been shown that in the temporal neocortex, the focal area has increased levels of NE, DA, dihydroxyphenylalanine (DOPA), 5-hydroxyindole acetic acid (5-HIAA) and homovalinic acid (HVA) (Goldstein *et al*, 1988; Louw *et al*, 1989).

Histamine also is believed to affect neurobehavioural disorders such as Alzheimer's disease, Down Syndrome, Attention Deficit Hyperactive disease, Epilepsy, Parkinson's disease etc. (Onodera and Miyazaki, 1999). CNS HA has been suggested to participate in seizure control. Intracerebroventricular (ICV) administration of HA decreased seizure susceptibility on electrically and pentylenetetrazol-induced

convulsions significantly and dose dependently, while centrally acting HA H1 antagonists such as pyrilamine (or mepyramine) and ketotifen antagonized the inhibitory effect of HA (Yokoyama *et al*, 1994 a,b).

Histaminergic neuron system is also believed to be involved in inhibition of seizures associated with febrile illness in childhood. The increase susceptibility to seizures during fever is hypothesized to be connected to the lack of increase in CSF HA in the febrile convulsive group (Kiviranta *et al*, 1995).

Upon stimulation of GABAergic system, decreases in cellular excitability is observed which leads to control of seizures. GABA receptor agonists have a wide spectrum as they antagonize not only seizures which are dependent on decreased GABA synaptic activity but also convulsion states which are apparently independent of alterations in GABA mediated events (Bartholini, 1985).

Seizure disorders have always been associated with a complex mixture of psychopathology. However never has sufficient attention been laid on the extent of structural damage that is associated with the disorder.

The observation, made by Bouchet and Cazauvieilh in 1825 of a palpable hardening and atrophy of the uncus and the mesial temporal lobe in patients with epilepsy did not attract much attention until Sommer (1880), some 50 years later, described neuron loss in a particular area of the pyramidal cells of the hippocampus in relation to epilepsy. However Sommer concluded that sclerosis to be the cause of epilepsy. The concept of the nature of ammonshorn sclerosis (Sommer, 1880) reached a new stage with the delineation of psychomotor epilepsy (Gibbs *et al*, 1948; Bailey and Gibbs, 1951; Earle *et al*, 1953; Penfield and Jasper, 1954; Falconer *et al*, 1955).

Subsequent studies (Seitelberger, 1969) have tried to reverse this hypothesis but still the older idea of epilepsy — a functional disorder without any consequent structural damage prevailed (Townsend, 1976).

It has been reported that recurrent limbic seizures caused a massive, delayed, and reversible reduction in levels of the kainite receptor mRNA in dentate gyrus; lesser decreases were found in pyramidal cell fields of hippocampus and superficial cortex (Gall *et al*, 1990).

These specific neurotransmitters take an active part in the control of brain activity. There may be a release of excitatory and inhibitory transmitters into the brain regions in response to nerve signals. The excitation passes down the cortex to widespread areas of brain and excites all the neurotransmitter systems differentially causing liberation of the neurotransmitters at different terminals which maintain the normal integrity of the brain functioning. Thus seizure activity is associated with wide range of local biochemical changes affecting various monoamines such as NE, DA, 5-HT and HA.

### Neuroprotection

The diagnosis of epilepsy is primarily made on history obtained from patient, relatives and witnesses. In the present era of diagnostic medicine with major reliance and dependence on modern equipment, there is a tendency to bypass or shortcut the

clinical history and examination. The most important aspect of the management of epilepsy is to remember that (1) whatever form of therapy is considered, epilepsy is one such disease where therapy once started is continued for a long time, at times for the whole life either continuously or with breaks. Most of the epilepsies with some exceptions are controllable but not curable. (2) The second part of importance is that epilepsy in majority of cases affects an individual at a young age in the early part of life when one starts acquiring education, vocational training, new career and social responsibility. In 75% of patients the epilepsy begins before the age of seventeen years. (3) The third point of great importance is that prescribing medicines is only one of the several facts of total management of patients with epilepsy. The different forms of treatment, which are available for the treatment of epilepsies, are (a) surgical (b) general (c) medical (Maheswari, 1993).

All forms of seizures should be treated with antiepileptic drugs, but an attempt should be made simultaneously to identify the difficult to treat other variations of the treatable epilepsies. Surgically treatable epilepsies should be identified. It should, however, be appreciated that only a very small number of patients are fit for surgical treatment.

Among dietary measures the Ketogenic Diet (high fat) has been found useful in severe childhood epilepsies. How it helps is not well understood. Also Ketogenic Diet is not very palatable.

Electromagnetic therapy has been detected around each individual person. However there are a few reports suggesting its influence on epilepsy. This needs further exploration.

Sleep disorders and epileptogenesis are very interlinked. Sleep deprivation have been used in the laboratories as provoking factors. Normalisation of sleep disorders may help the EEG abnormality and control the seizures in some patients.

If there are stress-induced seizures then it is sensible to reduce or avoid those stresses. Psychotherapy is effective in this direction in eliminating stress. Some change in life-style and occupation may also help in controlling seizures. Abstinence from alcohol and smoking and avoidance of toxic substances may at times help some patients.

Control of seizures by medication, though important, is only a part of total care of the patient. If social and environmental problems are not taken care of, then good control of seizures cannot be achieved. Better education, self-confidence and careful life-style restriction are important goals (Maheswari, 1993).

The aim of treatment with antiepileptic drug is to stop all seizures and to achieve complete suppression of all epileptic activity in EEG without producing side-effects for a duration long enough, so that the tendency to epilepsy ceases and treatment can be stopped. It is unlikely that this aim can be achieved. At present in a large number of cases, suppression of seizures is largely achieved by the development of newer and more effective drugs in the 20<sup>th</sup> century. Bromides were used for many years in the 19<sup>th</sup> century until phenobarbitone was made available for clinical use in the year 1912. Formerly, the aim of treating epilepsy was often regarded to be merely keeping the

sufferer free of convulsions, but the ever-growing contemporary hope for cure often entails a more vigorous and meticulous approach to drug therapy combined with social and rehabilitative measures. However, the aim remains restrictive and unrealistic in epilepsies due to progressive neuronal diseases and other development disorders. Impaired cognitive functions, i.e., learning and behaviour are effects of the condition causing epilepsy. Epilepsy itself may cause changes through its metabolic and neuro-transmitter interactions of excitation and inhibition. Although numerous drugs are available in the market for treatment of epilepsies, the existence and use of other forms of treatment in the management of epilepsies is itself an indication that all types of epilepsy are not easily controllable.

Herbal therapies are on the rise all over the world. Promising herbs like *Acorus calamus* (Hazra and Guha, 2003) and *Moringa pterygosperma* (Ray *et al*, 2003) can be developed into therapeutic agents. These drugs, once developed will be free from the evils of side effects, also they will be comparatively cheaper than the market available drugs.

The ideal antiseizure drug would suppress all seizures without causing any unwanted effects. Unfortunately, the drugs used currently not only fail to control seizure activity in some patients, but they frequently cause side effects that range in severity from minimal impairment of the CNS to death from aplastic anaemia or hepatic failure. The physicians who treat patient with epilepsy is thus faced with the task of selecting the appropriate drug or combination of drugs that best controls seizures in an individual patient at an acceptable level of untoward effects. It is generally held that complete control of seizures can be achieved in upto 50% of patient and that another 25% can be improved significantly. The degree of success is greater in newly diagnosed patients and is dependent on such factors as the type of seizure, the family history, and the extent of associated neurological abnormalities. All anticonvulsant drugs have also side-effects, trivial or major.

No system of the human body is spared by these drugs and common adverse reactions of commonly used antiepileptic drugs are given in

**Table 3. Commonly used antiepileptic drugs and their side effects**

Sl No.	Drug	Side effects
1	Phenobarbital (PHEN)	Drowsiness, aggression, irritability, poor concentration, hyperkinetic, impaired learning, skin rash.
2	Diphenylhydantoin (DPH)	Gum hyperplasia, nystagmus, ataxia, acne, hirsutism, coarsening of facial features, dysarthria, skin rash, involuntary movement.
3	Carbamazepine (CAB)	Skin rash, giddiness, double vision or blurred vision.
4	Sodium valproate (SV)	Nausea, vomiting, weight gains, loss or thinning of hair, tremors.

Sl No.	Drug	Side effects
5.	Ethosuximide (ETHO)	Vomiting, anorexia, euphoria, dizziness, headache, hiccup, anxiety, aggressiveness, inability to concentrate.
6.	Primidone (PRIM)	Sedation, Vertigo, dizziness, nausea, vomiting, ataxia, diplopia.
7.	Benzodiazepine	Lethargy, hypnotic, dysarthria, behavioural disturbance in children.
8.	Diazepam	Sedation, respiratory depression.
9.	Gabapentin	Somnolence, dizziness, ataxia.
10.	Nitrazepam	Sedation, drooling of saliva, dizziness.

However, searches in different directions have been conducted with the sole aim of helping the patient in controlling seizures. These various methods are undertaken not as the first sole method but as complementary to drug therapy.

## PARKINSON'S DISEASE

Parkinson's disease (PD) is a disease of Progressive Neurodegeneration. It was first described in 1817 by the physician James Parkinson. Previous to this, accounts of the symptoms were remarkably scarce, which led many researchers to theorize whether this disease may have been a product of the beginning of the early 19<sup>th</sup> century and the Industrial Revolution in England. Some speculated that, because certain environmental neurotoxins cause Parkinson's like ("Parkinsonian") symptoms, some contaminant in the new industrial environment may have increased its prevalence. The present concept is in favour of oxidative causation linked to environmental toxins.

Parkinson's disease is widespread in Westernized countries. The disease is highly age-dependent : it can manifest as early as the mid-30s, but becomes more common, past the age of 50, with 57 being the average age of diagnosis. It is visible as tremor in a limb, and as it progresses three other symptoms arise-bradykinesia (slowness of movement), rigidity (both "cogwheel" jerkiness and "leadpipe"/stiffness) and posture instability with impaired gait, associated with the stooped stance. Bradykinesia causes the patient to feel glued to the ground or to the chair in which they sit, and progressively erases body languages and facial expressiveness. The disease is not restricted to motor degeneration - as many as 35 percent of PD cases also develop dementia.

Parkinson's disease is now recognized to be a widespread degenerative illness

that affects not just the central nervous system, but also the peripheral and enteric systems. Formerly the disease was typecast as motor system degeneration, yet sensory fields, association areas, and premotor fields become damaged throughout the brain. Various lines of evidences has suggested that PD is primarily an oxidative disease, fueled by endogenous susceptibility and driven by the cumulative contributions of endogenous and exogenous (environment) oxidant stressors. The limbic, autonomic, and neurosecretory control fields (hypothalamus) all show micro-anatomic damage. At the cellular level, neuron death in PD is more systemic than previously assumed : non-invasive imaging recently demonstrated that the nerve supply to the heart degenerates in PD subjects. Biochemically, abnormalities of liver detoxification and mitochondrial oxidative phosphorylation also occur ( Kidd, 2000 ).

The pathological process that underlines PD typically is slow-paced but relentlessly progressive. The clinical symptoms tending to manifest relatively late in the pathological progression. Classically, the hallmark of PD has been degeneration of dopamine-producing neurons in the relatively small Substantia nigra (SN), most intensely localized in the zonal compacta.

Normaly, dopamine produced in the SN is moved to the caudate nucleus and the putamen, where it is involved in stimulating and coordinating the body's motor movements. In PD, neurons producing dopamine in the SN die, reducing the overall supply of dopamine and compromising the brain's capacity to effectuate movement. Curiously, dopamine-producing neurons outside the SN tend not to be affected, though many other neuronal types - glutamatergic, cholinergic, tryptaminergic, GABAergic, nora-drenergic, adrenergic - show "grievous cytoskeletal damage".

The characteristic pattern of nerve cell destruction in SN neurons appears to be linked to abnormalities that develop in the cytoskeleton. The pathognostic Lewy bodies and Lewy neurites are composed mainly of abnormal cytoskeletal neurofilament proteins. Neurons afflicted with Lewy formations remain viable for a relatively long period, but are functionally compromised and die prematurely. As a rule, projection neurons with long axons are more vulnerable than local circuit and projection neurons with short axons, tend to be spared.

### **A Broad Spectrum of Potential Etiologic Factors**

In spite of the extensive studies performed on postmortem substantia nigra from Parkinson's disease patients, the etiology of the disease has not yet been established. Nevertheless, studies have demonstrated that, at the time of death, a cascade of events had been initiated that may contribute to the demise of the melanin-containing nigrostriatal dopamine neurons. These events include increased levels of iron and monoamine oxidase (MAO)-B activity, oxidative stress, inflammatory processes, glutamatergic excitotoxicity, nitric-oxide synthesis, abnormal protein folding and aggregation, reduced expression of trophic factors, depletion of endogenous antioxidants such as reduced glutathione, and altered calcium homeostasis ( Mandal *et al*, 2003 ).

As with almost all disease states, a broad spectrum of both genetic and environmental factors have been suggested as contributing to the initiation and progression of PD. Aging is also implicated, with advanced age being the single most important risk factor for the disease.

## Role of Aging

Parkinson's disease is clearly age-dependent. Several of the neurodegenerative syndromes documented in the elderly — gait slowing, for example, resemble those seen in PD and may be prodromal for the disease while the inexorable downhill slide of Parkinson's disease is unmistakably a disease process. Aging undoubtedly contributes to PD progressing, perhaps because of its accumulative oxidative damage and steady decrease of antioxidant capacity.

## Heritability and Genetic Susceptibilities

There appears to be an inherited component to PD, and a number of family pedigrees with multiple cases of PD have been extensively studied. To date, seven loci on four chromosomes are reliably linked to PD and / or to neurodegeneration of the Parkinsonian type, not always with the presence of the Lewy structures. Non-familial PD subjects carrying a specific alpha-synuclein allele and ApoE4, have a 13-fold increased risk of developing PD. Parkin, localized to chromosome 6, causes early onset of Parkinsonism without the Lewy bodies that define PD. Most PD cases, however, do not have other affected family members and have no apparent familial contribution.

With the multiple of key studies now done, the preponderance of the evidence indicates the general PD population has no more than a mild genetic contribution. Still, overall absence of defined heritability does not necessarily rule out subpopulations with higher heritability, or subtle genetically - conditioned vulnerabilities. Various authors have described how various specific gene mutations and deletions might potentially contribute to PD. The genes involved could act with varying degrees of penetrance, or polygenically contribute to disease vulnerability with no single gene being wholly responsible. For early onset PD, the cumulative evidence is consistent with a strong heritability component.

Some individuals with PD have impaired liver detoxification. The P<sub>450</sub> IID6 enzyme, which was characterised based on its capacity to metabolize debrisoquine, was found to be dysfunctional more frequently in PD subjects than in non-PD controls. Of the PD subjects, those with very early onset (less than age 40) are most likely to have this problem. The gene for P<sub>450</sub> IID6 was localised to chromosome 22, and efforts are underway to develop it into a biomarker for early-onset PD, but this may not prove practical. Conducting such assays in vivo is expensive and laborious, and in the studies some of the medications being taken for PD may have complicated the outcomes.

Interestingly, P450 IID6 is present in the nigrostriatal system and the notorious Parkinsonian toxin, 1-methyl – 4-phenyl – 1,2,3,6-tetrahydropyridine (MPTP) is a substrate for this complex. MPTP came to the forefront of PD research in 1982, when drug addicts in Northern California began to develop severe Parkinsonism after intravenous injection of a synthetic heroin that was contaminated with MPTP as a by product of its synthesis. The identification of three genes and several additional loci associated with inherited forms of levodopa responsive PD has confirmed that this is not a single disorder. Yet, analysis of the structure and function of these gene products point to the critical role of protein aggregation in dopaminergic neurons of the

substantia nigra as the common mechanism leading to neurodegeneration in all known forms of this disease. The three specific genes identified to date — alpha-synuclein, Parkin, and ubiquitin C terminal hydrolase L1 are either closely involved in the proper functioning of the ubiquitin-proteasome pathway or are degraded by the protein-clearing machinery of cells. Knowledge gained from genetically transmitted PD also has clear implications for nonfamilial forms of the disease. Lewy bodies, even in sporadic PD, contain these three gene products, particularly abundant amounts of fibrillar alpha-synuclein. Increased aggregation of alpha-synuclein by oxidative stress, as well as oxidant-induced proteasomal dysfunction, link genetic and potential environmental factors in the onset and progression of the disease. The biochemical and molecular cascades elucidated from genetic studies in PD can provide novel targets for curative therapies.

The MPTP poisonings led to a serendipitous finding; that the symptoms resulting from exposure to MPTP very closely matched the many features of PD. This shifted the focus toward environmental factors as potential PD initiators or contributors. As the search progresses, a single toxic cause remains elusive but a role for environmental factors seems almost certain.

Although the contribution from environmental toxins cannot be denied, currently the cumulative evidence suggests PD is a multifactorial oxidative disease. The main causal, oxidative contributors indicated to date are : (1) measurable amplification of the endogenous oxidative load by constitutive impairments of mitochondrial energy transformations, (2) innate vulnerability of the brain's substantia nigra region to oxidative challenge, and (3) initiation or promotion by toxic exposure(s) that further deplete antioxidants. These factors combine to initiate a downhill course for the neurons of the SN and elsewhere in the brain, the end result of which appears to be a slow-acting yet long-term progressing, inflammatory process. This eventually results in the micro-anatomic degeneration and clinical symptomatology of Parkinson's disease.

The oxidative stress (OS) theory has implicated the involvement of reactive oxygen species (ROS) in both aging and age-dependent neurodegenerative diseases. The dopaminergic system is particularly vulnerable to ROS and dopamine (DA) itself can be an endogenous source of ROS. These results suggest that a neurochemical deficit and not cell loss *per se* within the nigrostriatal system underlies the motor behavioural deficits ( Cantutu-Castelvetre *et al*, 2003; Choi *et al*, 2003 ).

It is becoming widely accepted that the inflammatory response is involved in neurodegenerative disease (Castano *et al*, 2002). Elevated synaptic levels of dopamine may induce striatal neurodegeneration in L-DOPA-unresponsive Parkinsonism subtype of multiple system atrophy (MSA-P subtype), multiple system atrophy, and methamphetamine addiction ( Chen *et al*, 2003 ).

Parkinson's disease is most commonly a sporadic illness, and is characterized by degeneration of substantia nigra dopamine neurons and abnormal cytoplasmic aggregates of alpha-synuclein. Rarely, PD may be caused by missense mutations in alpha-synuclein (Dauer *et al*, 2002). It is characterized by focal microglial activation and progressive dopaminergic neurodegeneration in substantia nigra

compacts (SNc) (De-Giorgio *et al*, 2002) with no effective protective treatment characterized by a massive degeneration of dopaminergic neurons in the substantia nigra and the subsequent loss of their projecting nerve fibers in the striatum (Delgado and Genea, 2003).

The motor disturbances occurring in Parkinson's disease have been partially attributed to a hyperactivity of gamma-aminobutyric acid (GABA)-ergic nigral cells largely in the substantia nigra pars reticulata (SNr) secondary to the degeneration of dopaminergic nigrostriatal neurons. However, some aspects of this response remain unclear. Findings indicate a complex regulation of nigral GABAergic activity after nigrostriatal dopaminergic degeneration that probably involves local mechanisms, the nigro-striato-nigral loop as well as interhemispheric mechanisms whose anatomical basis remains unstudied (Diaz *et al*, 2003).

### Nitric Oxide and Parkinsonism

Nitric Oxide (NO), in excess, behaves as a cytotoxic substance mediating the pathological processes that cause neurodegeneration. The NO-induced dopaminergic cell loss causing Parkinson's disease has been postulated to include the following: an inhibition of cytochrome oxidase, ribonucleotide reductase, mitochondrial complex, I, II and IV in the respiratory chain, superoxide dismutase, glyceraldehyde-3 phosphate dehydrogenase; activation or initiation of DNA strand breakage, poly (ADP-ribose) synthase, lipid peroxidation, and protein oxidation; release of iron; and increased generation of toxic radicals such as hydroxyl radicals and peroxynitrite. NO is formed by the conversion of L-arginine to L-citrulline by NO synthase (NOS). At least three NOS isoforms have been identified by molecular cloning and biochemical studies: a neuronal NOS or type 1 NOS (nNOS), an immunologic NOS or type 2 NOS (iNOS), and an endothelial NOS or type 3 NOS (eNOS). The enzymatic activities of eNOS or nNOS are induced by phosphorylation triggered by  $Ca^{2+}$  entering cells and binding to calmodulin. In contrast, the regulation of iNOS seems to depend on *de novo* synthesis of the enzyme in response to a variety of cytokines, such as interferon-gamma and lipopolysaccharide. Selegiline, an irreversible inhibitor of monoamine oxidase B, is used in PD as dopaminergic function-enhancing substance. Selegiline and its metabolite, desmethylselegiline, reduce apoptosis by altering the expression of a number of genes, for instance, superoxide dismutase, Bcl-2, Bcl-xl, NOS, c-Jun, and nicotinamide adenine nucleotide dehydrogenase. The selegiline-induced antiapoptotic activity is associated with prevention of a progressive reduction of mitochondrial membrane potential in preapoptotic neurons. As apoptosis is critical to the progression of neurodegenerative disease, including PD, selegiline or selegiline-like compounds to be discovered in the future may be efficacious in treating PD (Ebadi and Sharma, 2003; Pang *et al*, 2000; Pardini, 2003).

Inflammation in the brain has increasingly been recognized to play an important role in the pathogenesis of several neurodegenerative disorders, including Parkinson's disease and Alzheimer's disease. Inflammation-mediated neurodegeneration involves activation of the brain's resident immune cells, the microglia, which produce proinflammatory and neurotoxic factors, including cytokines reactive oxygen intermediates, nitric oxide, and eicosanoids that impact on neurons to induce neurodegeneration (Geo *et al*, 2002; Liu *et al*, 2003).

Spiny neurons in the neostriatum are highly vulnerable to cerebral ischemia. Recent studies have shown that the postischemic cell death in the right striatum was reduced after ipsilateral dopamine denervation whereas no protection was observed in the left striatum after dopamine denervation in the left side. It is observed that after ipsilateral dopamine denervation, the depression of excitatory synaptic transmission and neuronal excitability therefore, might play an important role in neural protection after ischemic insult.

### Current Management of Parkinson's Disease

Classes of Prescription Drugs that can precipitate Parkinsonian Symptoms.  
(From Worst Pills ® Best Pills.)

**Antihypertensive, diuretic:** Diupres, Enduronyl, Hydropres, Regroton, Demi Regroton, Salutensin, Ser-AP-Es.

**Antihypertensive, non-diuretic :** Aldomet.

**Antidepressant :** Asendin, Aventyl/Pamelor, Desyrel, Elavil, Limbitrol, Ludiomil, Luvox, Norpramin, Paxil, Prozac, Sinequan, Tofranil, Triavil, Wellbutrin, Zoloft.

**Antipsychotic :** Compazine, Haldol, Mellaril, Navane, Prolixin, Risperdal, Stelazine, Thorazine, Triavil, Zyprexa.

**Others:** Reglan, Zyban.

Currently, PD is managed mainly through dopamine replacement therapy-Pharmaceutical agents aimed at replacing dopamine in the brain or mimicking its actions at dopamine receptors. Most commonly used is the dopamine precursor levodopa in combination with carbodopa (Sinemet (R) and Sinemet CR). The vast majority of patients experience benefits initially, but rarely do the benefits persist. Typically, after 2-5 years on levodopa drugs the patient's responses become erratic. Nausea is a constant threat, and dyskinesias develop that feature excessive and uncontrollable movements. Other adverse effects develop mental confusion, "freezing" and inability to move, dystonia, low blood pressure episodes, sleep disturbances, and hallucinations.

Adverse side effects usually pose a major ongoing challenge to the PD patient. For example, the combined effects of the disease and the drugs used to treat it produce sleep problems in an estimated 70 percent of patients and daytime hallucinations in about 30 percent. Levodopa is usually effective for motor symptoms at the beginning, but over time tends to cause motor fluctuations, dyskinesias, and other adverse side effects. These can become so disabling that surgical treatment becomes the only apparent option for restoring any quality of life.

Other drugs used for PD symptom management include amantadine (Symmetrel (R), selegiline (Eldepryl (R) deprenyl), dopamine agonists (bromocriptine, pergolide, pramipexole, ropinirole), and several anticholinergic drugs. All these have major adverse effects and generally are less effective than Sinemet (R) in suppressing symptoms. Tolcapone, an inhibitor of COMT (Catechol-O-methyltransferase, the enzyme which normally inactivates dopamine), became available in 1998. It caused several deaths from liver failure. Another COMT inhibitor-entacapone - was released in 1999 which was not liver-toxic but still caused dyskinesias, nausea, diarrhoea, abdominal pain, and urine discoloration.

As PD progresses, in addition to the adverse effects accruing from levodopa therapy the ever-worsening loss of dopamine neurons causes progressively crippling damage to motor control circuits throughout the brain. The control may shift, so the pathways that normally inhibit movement come to dominate those that activate movement. The increasing desperation of the patient can become the rationale for risky surgical intervention; for example, whether to remove inhibitory zones or to implant electrodes aimed at restoring a healthy balance of circuits.

Surgical destruction of brain tissue was tried prior to the advent of levodopa therapy, but produced inconsistent results. More recently, microelectrodes are being used to detect signals from individual brain cells, using these signals as "signposts" to arrive at more precise locations in the brain. Pallidal and subthalamic nuclear surgery can improve motor symptoms and levodopa-induced dyskinesias, but only unilateral pallidotomy is acceptable since the bilateral procedure carries unacceptably high risk. The unilateral procedure, however, probably the most common surgery for advanced PD, unfortunately does not allow for postoperative reduction in levodopa doses. Post-surgical mortality is 1–1.8 percent, risk of permanent neurological deficit is about five percent, and benefits tend to dissipate within 1–4 years.

Deep-brain electrical stimulation (DBS) by way of surgically implanted electrodes has the advantages over ablation of being regulatable and reversible. DBS also has been bilaterally performed in many patients with marked benefit and little permanent morbidity. DBS post-operative morbidity and mortality is less than for ablation, and the stimulation side effects are relatively mild. On the negative side, infection may ensue, mechanical failure occurs in 3–4 percent of cases, batteries must be replaced at regular intervals, and the device is expensive. On the positive side, successful bilateral stimulation can allow medication dosing to be reduced, providing the patients a better quality of life. Although results from randomized studies are not yet available, surgeons who have done both ablation and DBS agree that DBS is better for the patient.

Epidemiological and clinical studies provide growing evidence for marked sex difference in the incidence of certain neurological disorders that are largely attributed to the neuroprotective effects of estrogen. Thus there is a keen interest in the clinical potential of estrogen-related compounds to act as novel therapeutic agents in conditions of neuronal injury and neurodegeneration such as Parkinson's disease.

Obviously, the need to broaden the options for therapy in Parkinson's disease is urgent. The urgency compels renewed focus on the etiology and pathogenesis of the disease. Deeper scientific understanding of PD would lead to better preclinical detection and prophylaxis, validation of biomarkers, confirmation of genetic and environmental risk factors, and more prolonged symptom control with fewer adverse effects.

## SUMMARY

Parkinson's disease (PD) is characterized pathologically by preferential degeneration of the dopaminergic neurons in the substantia nigra pars compacta (SNc). Nigral cell death is accompanied by the accumulation of a wide range of poorly degraded proteins and the formation of proteinaceous inclusions (Lewy bodies) in

dopaminergic neurons. Mutations in the genes encoding alpha-synuclein and two enzymes of the ubiquitin-proteasome system, parkin and ubiquitin C-terminal hydrolase L1, are associated with neurodegeneration in some familial forms of PD (McNaught *et al*, 2003). This disorder is characterized by tremor, muscular rigidity, difficulty in initiating motor activity, and loss of postural reflexes.

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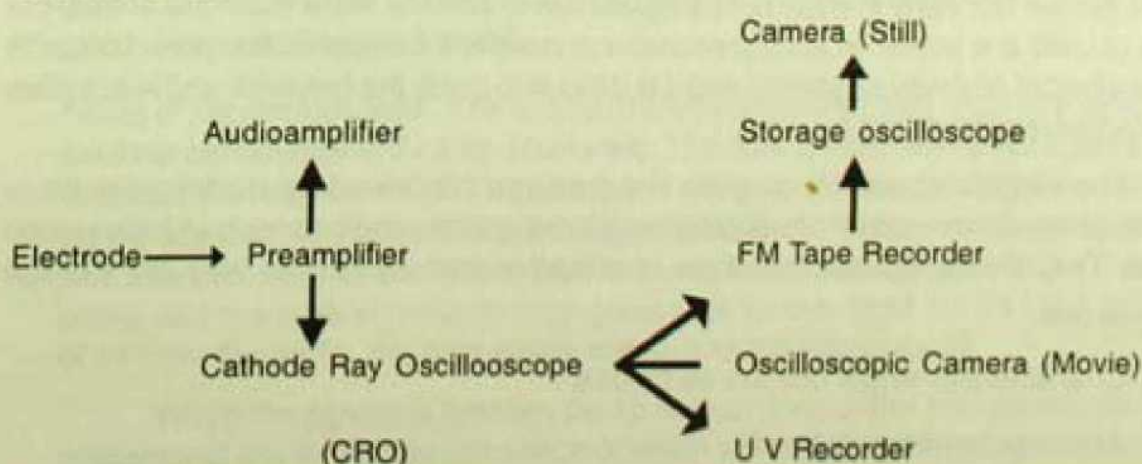
# Electrophysiological Techniques Utilized in the Study of Biological Systems

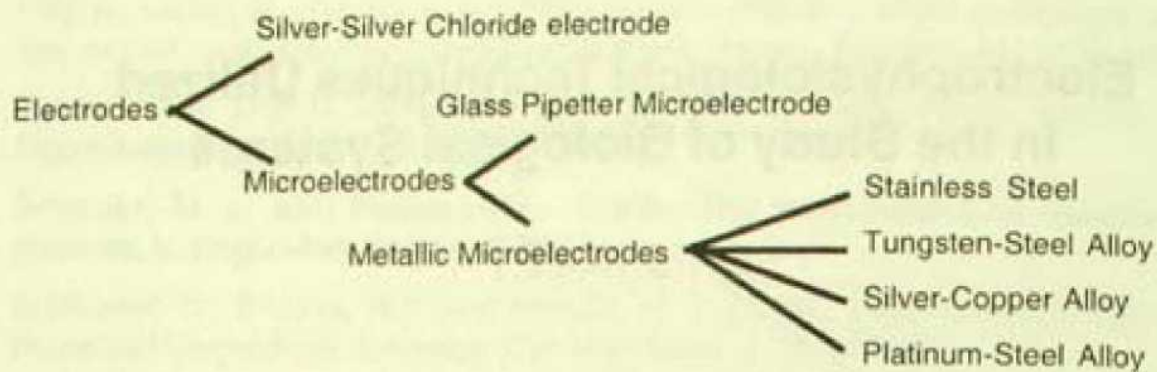
DR. J. KOLEY

**I**n the living organisms, the life, process is continued through various electrical events. Electrical potentials exist across the membrane of essentially all cells in a living body. Some cells like muscle and nerve are excitable and capable of generating electro-chemical impulse at their membrane and these can transmit signals. Other cell types like glandular cells, the changes in membrane potential may play important role in control of their functions. The information about both internal and external environment and response from the centre to combat the situation is performed through these electrical events. There are different methods to study the properties and behavior pattern of the centre, pathway and receptor or motor organs. The electrophysiological technique is the most effective and accurate method for finer studies.

Before the advent of the single fibre technique, multi fibre techniques were in practice in order to investigate the electrical properties of nerve cells. In multi fibre technique where there are multiple active fibres with different amplitude and wave pattern and thus, it is difficult to study the behavior and reactivities of individual nerve or receptor. In single fibre technique, there is only one active unit or nerve, so it is most suitable to study the behavior (conduction velocity in case of a nerve) and reactivities (natural stimulus in case of receptor) of the neuron. Single Fibre Technique were developed by Paintal in 1953. Single neuronal study in peripheral or central nervous system is also possible through microelectrodes.

The electrophysiological technique for single nerve/neuron studies is comprised of a circuit like





## SINGLE FIBRE TECHNIQUE

**Silver Silver Chloride Electrode :** This technique was developed by Paintal in 1953. In this technique bipolar Silver-Silver Chloride electrodes are used. Pure silver wires of about 1 mm thickness are first soldered at the tip of two metal net shielded cables, which are attached firmly to a flexible electrode holder. The electrolytic chlorination of the pure silver wires is carried out with a very weak current density (1-10A/m.sq) and current is passed through for overnight to deposit AgCl over the silver electrode. The nerve to be studied is cleared carefully from the surrounding tissue and a small pool is made around the nerve. The pool is filled with liquid paraffin. The nerve is placed over a black ebonite dissecting plate that improves the visibility of the fibre under microscope and kept emerged in the liquid paraffin pool (nonconductor) to avoid drying. The nerve is desheathed with a micro knife under a Binocular Stereoscopic Dissecting Microscope. The central/peripheral cut end of the desheathed nerve is split into fine strands with the help of watchman's forceps/twizlers. The split nerve strands are placed one by one over the silver silver chloride electrode, which is kept immersed in the liquid paraffin pool. The electrode is connected to the differential preamplifier, which in turn is fed to the CRO and also to an audio amplifier in parallel connection for monitoring the sound of the nerve activity. Initially multiunit activity may be obtained and after repeated splitting single unit activity may be achieved.

Single fibre technique does not mean isolation of single fibre or single axon. Single unit activities are studied / recorded from a strand of nerve fibres in which one unit or axon is active. Similarly in multi fibre preparation or activity more than one or multiple axons or units are active. In such preparations there are multiple action potentials with different height and wave patterns and it is difficult to study the behavior and reactivities of individual receptor or axon.

The single unit activity may be checked and confirmed by studying the wave pattern of the action potentials (same height) on oscilloscopic screen at high sweep speed. This check up can be done in a better and confirmed way on storage oscilloscope.

Characters of single unit are as follows :

Action potentials will be of

same height

- same wave pattern (shape and contour)
- on increase of stimulus strength, frequency of discharge will increase but not the height.

Hence action potential obeys All or None law.

Single fibre preparation help in the study of

- Distribution / localization of Receptors
- Behavior and reactivities of the receptor
- Conduction velocity of the nerve fibre
- Nature of the nerve fibre (diameter, etc).

**Microelectrode Technique** : Intracellular and extracellular neuronal activities of one single neuron or axon in the central nervous system or peripheral nerve can be recorded with microelectrode.

There are two types of microelectrodes

- Glass pipette microelectrode
- Metal wire microelectrode

### **Glass Microelectrode Technique**

Pyrex or Corning glass tubes of 2-3 mm outer and 1.5 - 1.8 mm inner diameter and about 8cm long are used for preparation of glass microelectrode. Such glass tubes of desired length are washed with distilled water and then dried. Earlier glass microelectrodes were prepared over flame by hand pulling. Now Microelectrode Pullers are available. The top end of the glass tube is fixed at the upper hinge of the puller and passed through the heating coil. The lower end of the glass tube is fixed to a free clamp attached with a weight at the bottom. The heating coil is then put on and the glass tube starts to soften. When the glass is soft enough, the weight attached at bottom pull the glass tube to form two uniform micro tipped glass pipettes electrodes. Now similar two glass micro pipettes are formed.

**Filling of the micropipette** : The empty micropipettes are fixed around a wide cork with a rubber band keeping their tip downward. The cork is fixed with a solid glass rod at the centre of the upper surface. The upper end of the rod is fixed at the bottom of a rubber cork that set air tight of a wide mouthed glass bottle. The bottle is filled partially with 3M KCl solution so that the glass pipettes are half dipped in the KCl. The bottle is connected to a suction pump through glass tube for overnight (18-24 hrs.). With suction of air from the bottle, the fluid slowly enters into the micropipette.

When the pipette is full then the tip is examined under microscope to check the presence of any air bubble (presence of which will increase the tip resistance) and also the tip diameter (ideal one is  $1\mu\text{m}$ ). The ideal tip resistance should be 5-10 M $\Omega$ . The slope with which the electrode narrows down is important - greater this taper, lesser will

be the puncture opening for the same depth of insertion.

**Quick fill glass microelectrode** - During 1980s, quick fill type of corning microelectrode was developed. The tube contains a fine glass filament inside. The microelectrodes prepared with such quick-fill glass tubes contain the glass filament, which helps in filling up of the pipette with 3 M KCl by capillary action. Having the microelectrode of desired shape and size the quick fill type can be filled with ordinary syringe only and the tip resistance should be 5 - 10 MΩ.

**Metallic microelectrode** : Metal microelectrodes are suitable for extra and intracellular recording of action potentials or synaptic activity but cannot be used for DC potential recording because of polarization, contact potential or poor stability. Low impedance, low noise and ruggedness are the advantages of metal microelectrodes.

#### *Preparation of the metal microelectrode*

Initially wire (1 mm) is straightened by passing electric current (the wire will be hot due to high resistance and be straightened). The straight wire is cut into pieces of 5-6 cm. long and washed thoroughly with distilled water and dried. The metal microelectrodes are prepared by utilizing the process of electrolysis in which the cut pieces are fixed radially on a metal ring rotating on its axis. The ring is fixed over a container filled with 10 to 20% HCl. One rotation of the wheel electrodes are dipping into the acid solution completing circuit and then coming out gradually. So the tip of the wires are facing the electrolysis for maximum time and resulting maximum narrowing. On examination under microscope when the tip diameter is 1 μm - indicates the end point. The electrodes are washed thoroughly with distilled water, dried and now ready for insulation.

#### *Insulation of the metal microelectrode*

Various substances like nail varnish to special varnishes are used for metal microelectrode insulation. The whole electrode together with the several cm. of the needle is immersed in the varnish then slowly pulled out. The slow backward movement prevents formation of drops along the electrode. Mechanical devices may also be used for pulling out the electrode uniformly.

In another method the tip leaves varnish fast. The electrode is completely submerged and just before pulling it out, air is blown on the surface of the varnish and thus a fine layer contributes insulation of the tip, which due to surface tension remains uninsulated. The varnish may be dried in air or oven. Before use it is necessary to determine the size of the uninsulated tip and flaws in insulation if any along the length of electrode. If the electrode's shape is satisfactory under microscope and the tip resistance is 5 to 15 M, it is ideal for utilization.

#### **Recording of unit activity from central neurones**

Generally stereotaxic apparatus is utilized for recording unit activity from central neurones, particularly brainstem, hypothalamus, cerebellum, cerebrum etc. There is stereotaxic co-ordinates which help in placing the microelectrode in the desired nuclei in the central nervous system. For recording unit activity with microelectrode the electrode is connected to cathode follower which is in turn connected to the CRO via

preamplifier. In each case electrode position is to be checked by putting a marker at the side of the electrode tip and then studying the serial sections of the brain tissue after the experiment.

### PATCH CLAMP - VOLTAGE CLAMP TECHNIQUE

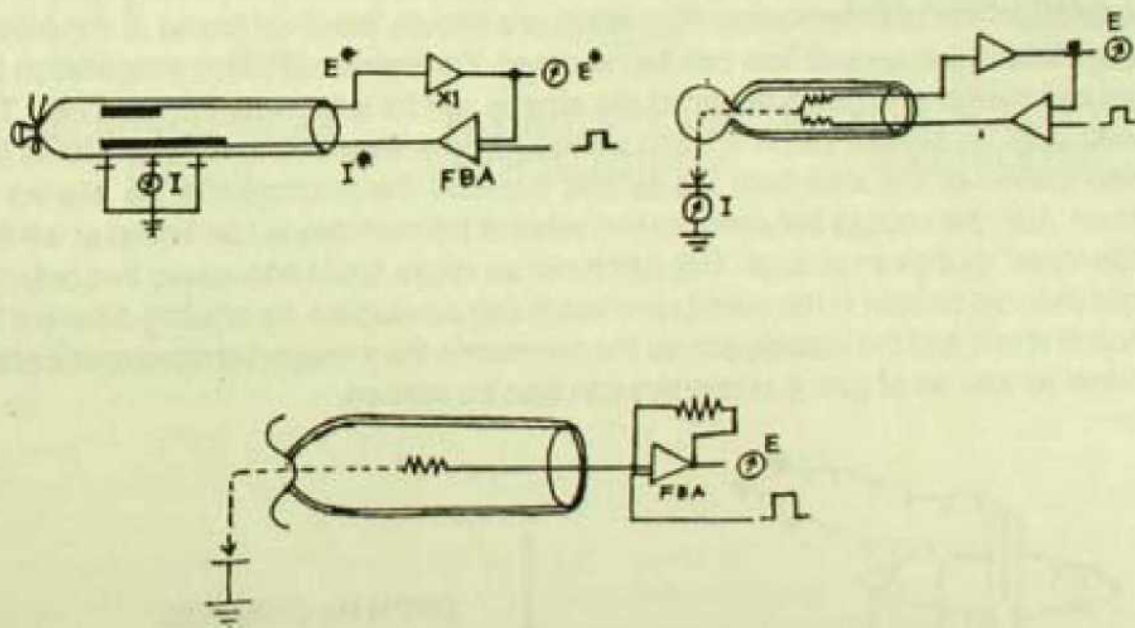
The electrical excitability in terms of ionic permeability change in excitable tissue or cell membrane is derived by patch clamp or voltage clamp technique. Voltage clamp means control of the membrane potential electrically. The two advantages of voltage clamp over the conventional voltage recordings are:

- The recorded current is carried by the ions and gives a direct measure of ionic permeability changes of the membrane.
- The gating of Na, K, Ca channels depends on membrane potentials.

So the manipulation of this variable permit the kinetic of gating to be controlled and analysed.

The first voltage clamp was tried by K.S. Cole (1947) with squid giant action (Axial wire).

There are many voltage clamp techniques designed for different types of cells.



- $E^*$  - Voltage electrode - intracellular potential is detected by  $E^*$
- $I^*$  - Transmembrane current is applied intracellularly by the electrode  $I^*$
- $I$  - The bath is grounded through a current measuring system  $I$
- $X1$  - Unity gain follower
- FBA - Feed back amplifier

### E - Voltage indicating circuit

FBA - supplies the membrane current required to keep the membrane potential equal to the command voltage shown as the square wave (  $\Omega$  ) pulse.

### Patch clamp technique for recording ion current flow through a single (Not Cold) channel (Neher et al 1981)

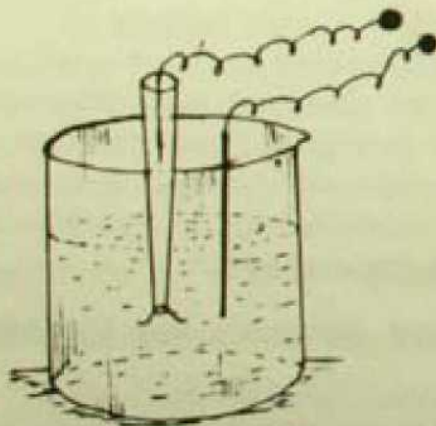


Inside out

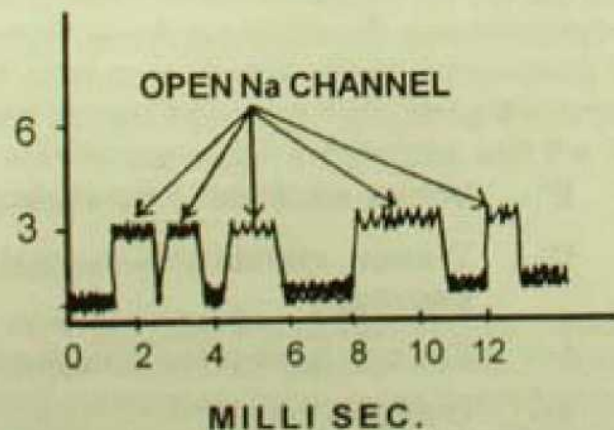


Outside out

By using patch clamp technique it is possible to record/study the ion current flow through a single channel. A micropipette having tip diameter about 1 to 2  $\mu$  is abutted against the out side of a cell membrane and suction is applied inside the pipette to pull the membrane slightly into it. This creates a seal where the edges of the pipette touch the cell membrane. The result is a minute 'patch' at the tip of the pipette through which the current flow can be recorded. For inside out patch preparation the small cell membrane patch at end of the pipette can be torn away from the cell. The pipette with its sealed patch is then inserted into a free solution. This allows the concentration of the ions both inside and out side the micropipette be altered as desired. Also the voltage between the two sides of the membrane can be set at will that is "Clamped" to a given voltage. This patch can be made small enough so that only one single channel protein in the membrane patch can be studied. By altering different ion concentrations and the voltage across the membrane the transport characteristic of the channel as well as of gating properties can also be studied.

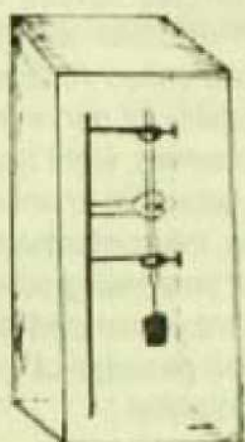


PICO AMPERES

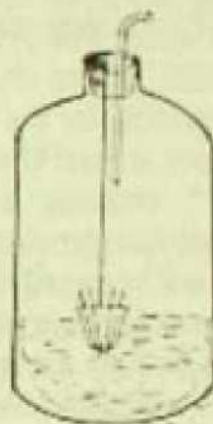


### Open and closed steps of gated channels

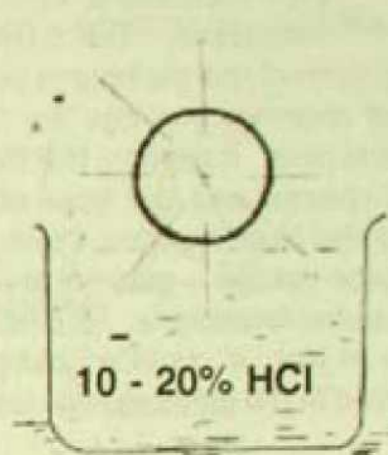
The study of the electrical current flow through a single Na channel shows that the channel conducts current either all or none. That is, the gate of the channel snaps open and snaps close, each snapping events occurring within a few millionths of a second. These demonstrate the rapidity with which conformational changes can occur during opening and closing of the protein molecular gates.



**MICRO ELECTRODE  
PULLER**



**FILLING OF GLASS  
MICROELECTRODE**



**METAL MICROELECTRODE  
ELECTROLYSIS**

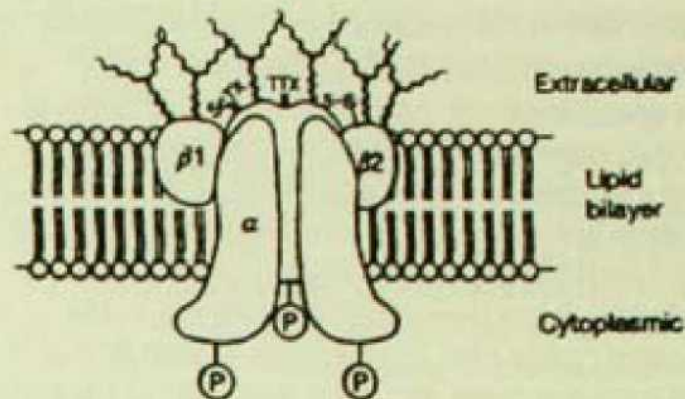
# Voltage – Gated Channels and Excitation of Nerve Fibres

PROF. TUSHAR KANTI GHOSH

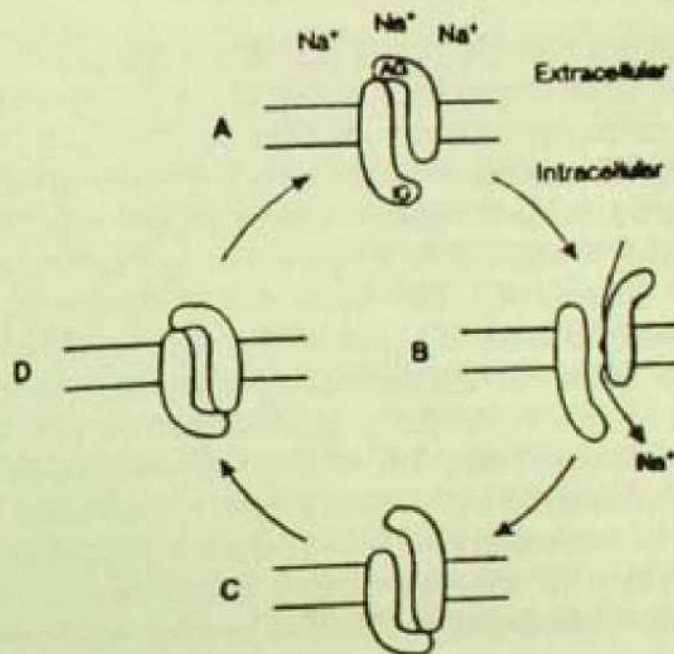
**E**xcitabile cells like nerve and muscle cells contain some channel proteins on their membrane which are sensitive to the voltage changes across the membrane. The channel within this protein may open or close with the fluctuation of the membrane potential. These channel proteins are called voltage – gated channels and they are responsible for the electrical excitability of nerve and muscle cells. It appears that there are at least three types of  $\text{Na}^+$  channel, eight types of  $\text{K}^+$  channel and four types of  $\text{Ca}^{++}$  channel. Some voltage – gated  $\text{Cl}^-$  channels have also been reported. During electrical stimulation of nerve fibre, the conformation of some voltage – gated channels are changed due to change of potential gradient across the membrane. As a result the channels within this protein may open and ions such as  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{++}$  can pass through the membrane. These passage of ions resulted in the generation of local potential and finally the action potential.

## SODIUM CHANNEL

Sodium channel is by far the best known of the voltage controlled channels. These proteins can specifically bind with the tetrodotoxin (TTX), a neurotoxin derived from fish belonging to the family Tetrodontidae, the best known example being the Japanese puffer fish. On the basis of this specific binding of TTX, the sodium channel can be isolated. A 260 KDa glycosylated sodium channel protein has been isolated from the electric organ of eel. The sodium channel isolated from mammalian brain shows a large  $\alpha$  – subunit (260 KDa) and two other smaller polypeptides,  $\beta_1$  and  $\beta_2$  subunits (36 KDa and 33 KDa)(Fig.1). The channel is present in the  $\alpha$ -subunit and  $\beta$ -subunits are shown to be modulating the channel properties of  $\alpha$  – subunit. The Japanese group led by Shusaku Numa in the year 1984 applied recombinant DNA techniques to clone and sequence the  $\alpha$ -subunit of eel electroplax. This sodium channel consists of a single run of 1820 amino acids containing four homologous domains of approximately 300 amino acids each. Hydropathy analysis indicates there are six membrane spanning helices (S1, S2, S3, S4, S5 and S6) within each 300 residue domain. S4 is found to contain a number of positively charged residues and is believed to be the “voltage sensor”. In between S5 and S6, an antiparallel pleated sheet structure (designated H-5) is present in each domain. This H-5 is believed to line the pore and confer ion selectivity. Inactivation of  $\text{Na}^+$  channel probably occurs by the



**Fig. 1.** Schematic cross section of brain sodium channel in membrane.  $\alpha$ ,  $\beta_1$  and  $\beta_2$  - subunits are heavily glycosylated on their extracellular surfaces. The  $\alpha$ -subunit has binding sites for tetrodotoxin (TTX) and  $\alpha$ -scorpion toxins (ScTX).



**Fig. 2.** Conformational cycle of a sodium channel. AG : Activation gate. IG: Inactivation gate

- A: "Resting" membrane sodium channel in closed condition
- B: Depolarized membrane sodium channel in open condition
- C: After about 1msec the IG closes
- D: Membrane returns to resting voltage and AG closes. IG gate reopens slowly then (about 1.5 msec ) and the channel returns to original conformation.

membrane protein exposed to the inside of cell. From the experiments of Armstrong and Bezanilla (1974) it appears that inactivation does not occur if protease is injected into the cell. It was speculated that the mechanism could work like a ball and chain. The globular part of the protein acting as a ball could plug the inside mouth of the pore while the chain being a flexible part could allow the ball to flop in and out of the mouth, depending on the voltage across the membrane. The short intracellular section between homologous domains 3 and 4 is responsible for inactivation of the channel and is acting like a ball and chain model. It has been found from the patch-clamp technique that the sarcolemma  $\text{Na}^+$  channel opens after 10 mV depolarization above its resting potential. Each channel shows a square pulse of current flow suggesting a sharp "on" and "off" mechanism. The inward direction of the current across the membrane indicates the flow of  $\text{Na}^+$  ions from outside to the inside of the membrane down their concentration gradient (Fig. 2). The average opening time of one channel is 0.7 msec and 1.6 pA current flows through the channel during open phase. The inward current of membrane ( $I_m$ ) during depolarization is due to activation of many sodium channels and is called sodium current ( $I_{\text{Na}}$ ).

### POTASSIUM CHANNEL

$\text{K}^+$  channel was isolated first from the *Drosophila*. A well characterized mutation in *Drosophila*, the shaker mutation affected  $\text{K}^+$  channel. cDNA clones were isolated from the shaker region and it can be expressed in xenopus oocyte system, where it shows all the physiological and pharmacological properties of  $I_K$  channel. It appears from the biophysical study that there are different types of voltage gated  $\text{K}^+$  channels. They have been termed the fast or early  $\text{K}^+$  channel ( $K_v$ ), the delayed rectifier  $\text{K}^+$  channel ( $K_v$ ), the serotonin-dependent  $\text{K}^+$  channel ( $K_s$ ),  $\text{Ca}^{++}$ -dependent  $\text{K}^+$  channel ( $K_{\text{Ca}}$ ), G-protein-linked muscarinic potassium channel ( $K_{\text{ACh}}$ ) or GIRK-1 etc. The fast  $\text{K}^+$  channel is formed by four units. Each unit is a polypeptide having molecular weight of 70 KDa and consists of 616 amino acids. Hydropathy analysis shows that there are six transmembrane (6 TM) helices (S1-S6). The stretch of amino acids between S5 and S6 forms an antiparallel pleated strand (H-5) like the  $\text{Na}^+$  channels. This region is believed to line the pore formed within the four unit cluster of the channel protein. The S4 region is arginine rich as in  $\text{Na}^+$  channel. It appears that N-terminal sequence acts as a kind of "ball and chain", inactivating the channel by plugging the pore.

During the recovery phase of nerve action potential some  $\text{K}^+$  channels are opened through which  $\text{K}^+$  move from inside of the cell to outside. As these channels open  $2\mu$  sec after depolarization of the membrane, they are called delayed  $\text{K}^+$  channels ( $K_v$ ). Delayed  $\text{K}^+$  channels remain open much longer while the membrane is depolarized. The current flowing through  $K_v$  during generation of action potential of nerve is called  $I_K$  and contributes to outward portion of  $I_m$ . This  $K_v$  channel also shows 6 TM (transmembrane), structure. The ATP sensitive  $\text{K}^+$  channel ( $K_{\text{ATP}}$ ) and GIRK-1 are also formed by four protein subunits. But each unit is showing 2 TM structure instead of 6 TM structure in other  $\text{K}^+$  channels.

### CALCIUM CHANNEL

There are at least four major types of  $\text{Ca}^{++}$  channels (L, N, P, T types). All of these  $\text{Ca}^{++}$

channels are formed by one polypeptide chain which has four homologous domain as in  $\text{Na}^+$  channels. The homologous region shows 6 TM structure with H-5 loop in between S5 and S6.

The three voltage-gated channels,  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{++}$  show a similar plan of structure. They have H-5 pleated structure which forms the line of pore and S4 segment containing positively charged residues. H-5 is involved in ion selectivity and S4 acts as a "voltage sensor". Ions do not flow through membrane pores like water through a tube. They proceed in single file, from one binding site to next. Substitution of certain amino acids in the H-5 region altered channel conductivity. Mutation studies of  $\text{Na}^+$  channels in H-5 region shows when  $\text{Lys}_{1422}$  and  $\text{Ala}_{1714}$  are substituted by negatively charged Glu residues, the ion selectivity of the channel is dramatically changed from  $\text{Na}^+$  to  $\text{Ca}^{++}$ . It has been suggested on the basis of mutation studies that S5 and S6 also contribute to the wall of the pore. The opening of the voltage-gated channel due to changes of voltage across membrane is proceeded by a small current which is called gating current. There is also a gating current ("off" gating current) before inactivation of the channel. In the S4 segment every third residue is positively charged. In an  $\alpha$ -helical conformation all these charged residues will be projecting in approximately the same direction. These positive charges are neutralised by negative charged amino acid side chains from other parts of channel protein. The whole structure is held in position by the voltage across the membrane. It has been hypothesised that during depolarization of the membrane the negative potential on the internal surface of the membrane is reduced and as a result the helical structure of S4 "screws" outward by 5 Å and through 60°. This transfers one positive charge to the outside. In an alternative hypothesis the voltage gated channel proteins are compared with superionic conductors which have conductivities comparable to those of ionic solutions. Hence a pore is not envisaged here within channel. In the open state channel protein conducts ions by becoming a metalloprotein that contains a chain of permeant ions loosely bound to sites along a transmembrane path. The electrochemical gradient across the membrane forces the ions to hop from site to site. In this model opening and closing states of the channel protein are compared to the properties of ferroelectric materials that exhibit a spontaneous electric polarization reorientable by an electric field. This ferro electric-superionic transition hypothesis explains channel opening simply as a shift in the transition temperature induced by the reduction in the electric field due to a depolarization.

## CHLORIDE CHANNEL

The voltage sensitive chloride channels have been identified. There are at least three  $\text{Cl}^-$  channels: chloride channel ( $\text{Cl}_c$ ), chloride-nucleotide-modulated channel ( $\text{Cl}_n$ ) and phospholemman. The  $\text{Cl}_c$  consists of two 89 KDa subunits. Each subunit is a polypeptide containing 805 amino acids and shows 12 TM structure. The voltage sensor and ion selectors have not been identified. The  $\text{Cl}_n$  channel protein contains 235 amino acids and has one membrane embedded domain which has four amphipathic  $\beta$  strands. Phospholemman is a small polypeptide (72 amino acids) with one transmembrane domain. The opening and closing mechanism of these channels are not clearly understood.

In the excitable tissue the generation of action potential is due to the opening and closing of the voltage – gated  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{++}$  channels. The conductance of the membrane is changed during stimulation due to changes of voltage-gated channels. As the channels are of different structure and biophysical properties, the nature of unitary current is not similar in all the channels. Moreover, there are different varieties of each ion channels and some of these are activated during excitation. The small unitary currents of many single channel are summated to form the large membrane current. The membrane potential is changed as a result of this current flow. In this respect action potential may be considered as an orchestra of ionic channels.

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# Male Reproductive Toxicology — New Perspective in Life Science

DR. AMAL ROY CHOWDHURY

## I NTRODUCTION

The survival of any species depends upon the integrity of its reproductive system. Male reproductive system consists of testes, which produces sperm, and the accessory organs i.e. epididymis, seminal vesicle, vas deferens, prostate that produce secretory products and form seminal plasma where sperm remain suspended<sup>1, 2</sup>. Since early '70's due to rapid industrialization and overgrowing urbanization, the toxic effects of these environmental chemicals on male reproduction have become a major health concern in the globe<sup>3, 4</sup>. Studies revealed that enormous and careless use of these chemicals caused detrimental effects on different organs but broad-spectrum irreversible toxic actions at cellular and molecular levels were observed mainly on reproductive system of human and experimental animals<sup>5</sup>.

### CHEMICAL INDUCED TESTICULAR TOXICITY

The potential toxicity of various chemical i.e. pesticides and heavy metals caused alteration in sperm morphology, count, motility as well as biochemical disruptions of enzymes and hormones.

#### Pesticide : Lindane Induced Testicular Injury

Lindane, the disomer of Benzene hexachloride, was used as DDT-resistant pest controller and in horticulture, forestry, public health and veterinary science. Although it was banned in developed countries but developing countries like India, Pakistan, Sri Lanka are still using due to economic reason. The embryotoxic and teratogenic effects of Lindane were documented in IARC monograph in 1971, but now extensive studies on farmers and on experimental animals revealed its spermicidal activity. Biopsy taken from the testicular tissue of the occupationally exposed farmer to Lindane revealed the disintegration of germinal cells and peritubular membrane (PTM), presence of pyknotic nuclei of Leydig cells and intratubular edema. Maximum deposition of sudanophilic materials in testicular tissue indicated high lipid concentration due to impaired steroidogenesis in Leydig cells<sup>(6)</sup>.

Lindane induced injury is predominant in epididymis, which causes decline in

the rate of sperm maturation and significant decline in succinic dehydrogenase (SDH), Hyaluronidase, 3bD<sup>5</sup>HSD, ACPase and Testosterone<sup>7</sup>. Lindane caused gradual accumulation of free radicals due to sharp decline in the scavenging activity of cytosolic Superoxide dismutase (SOD) and catalase and decrease of ascorbic acid content together with an increase in the levels of lipid peroxidation and H<sub>2</sub>O<sub>2</sub>, which caused reduction of epididymal sperm number with a dead and damaged spermatozoa (SZ) having anomalous head<sup>8</sup>.

Studies also revealed the protective role of vitamin A against Lindane as withdrawal of vitamin A from diet exhibited all the spermicidal functions of Lindane stated above, whereas supplementation of vitamin A alter withdrawal of pesticide in diet accelerated the recovery and restored spermatogenesis and steroidogenesis in deficient group, which demonstrate greater susceptibility of male reproductive system to Lindane toxicity during vitamin A deficiency<sup>9</sup>.

### Effect of Heavy Metals

(i) **Lead**: Lead is widely used in acid battery plant refinery, smelter, fuel combustion industry, printing press and in automobile exhaust where tetraethyl lead act as anti-knocking agent. Toxicity is manifested by deposition of lead in testes, epididymis, vas deferens, seminal vesicle and decreased volume of ejaculate, total sperm count and alive spermatozoa (SZ), retarded activity of sperm ie. reduced motility as well as prolonged latency of sperm melting both in exposed person and experimental animals<sup>16,17</sup>. Study with male CF-1 mice indicated significant decrease in epididymal sperm count at low dose (0.25% via drinking water), decreased motility and increased incidence of teratospermia at higher dose (0.50%) along with inhibition of post-meiotic cells mainly pachytene spermatocyte (SC) and ST, detachment of germinal cell layer from basal membrane, atrophy of Leydig cells plus interstitial edema and low density of seminal plasma<sup>18</sup>.

Low activity of ATPase and adenosine mono phosphatase (AMPase) at the basement membrane (BM) of seminiferous tubules was observed in rats exposed to lead at doses of 6 mg/kg ip over a period of 90 days<sup>17</sup>. The exact reason behind the event is not fully known, but very recently scientists suspected that lead interfered with the enzymes which contain (-SH) group or with the intervening redox systems and tissue respiration<sup>18</sup>. Low fructose content decreased activity of SDH and alkaline phosphatase (Alk.Pase) in seminal plasma were observed among the workers of printing press exposed to lead more than 8 h/d over a period of 10 yrs.<sup>16</sup>. Moreover, workers occupationally exposed to lead exhibited moderately high blood lead levels associated with sexual disorders like decreased libido that was followed by an increased frequency of astheno-, hypo- and teratospermia<sup>19</sup>. Lead also attacked the K<sup>+</sup>- channel in sperm tail membrane or interfered with early signal transduction after membrane depolarization<sup>20</sup>.

(ii) **Cadmium**: Cadmium released from tannery, smelter, battery crushing and preparing unit. The action of cadmium is spermatogenic stage specific and continuous<sup>15</sup>. High doses of cadmium-chloride caused rapid testicular edema, hemorrhage and necrosis. Cadmium exerted deleterious effect on the vascular structure of testis that may be the result of varying degrees of cadmium induced ischemia. Degeneration of testicular

tissue after different doses of cadmium exposure caused rupture of blood vessels<sup>16</sup>. Electron microscopic observations revealed that DNA fragmentation in mouse testicular tissue may be induced by cadmium, but chelating agent selenite exhibited a protective role<sup>17</sup>.

Zinc plays an important role in maintenance of structure of super oxide dismutase (SOD), which scavenges the free radicals and maintain appropriate spermatozoal milieu<sup>18</sup>. Cadmium replaces  $Zn^{+2}$  leading to enzyme structural distortion, which was manifested by reduced activity of SOD. Viability of SZ was also reduced in cadmium exposed groups<sup>18</sup>. Moreover, cadmium directly or indirectly targets GSH-Px, which catalyzed the destruction of  $H_2O_2$  and lipid hydro peroxides by reduced glutathione (GSH) and protecting the membrane lipids from peroxidative damage in a highly oxidative stress condition, the ultimate result is membrane degeneration of spermatozoa leading to abnormal and dead sperm in semen. Selenium (Se) and vitamin E exhibited protective role against LPX because Se maintained GSH-Px activity in SZ and seminal plasma. Also high vitamin E concentration in SZ was associated with reduction in its susceptibility to LPX<sup>19</sup>. Metallothionem (MT) - a low MW metal binding protein, plays an important role in cadmium detoxification because of its high cysteine content. Cadmium was bound to MT very tightly so that the metal may no longer be available to produce toxicity<sup>18</sup>.

(iii) **Chromium**: The nephro-and dermatotoxic heavy metal chromium are widely used in refractory, pigment and stainless steel factory, tannery, welding, engraving and photo processing unit. It caused severe reproductive injury in the exposed persons. Industrial workers exposed to chromium over 25 months for 6 h daily showed high metal level in blood and semen. The welding fumes contain high percentage of chromium and so the welders exposed to smoke generated by welding, suffered from an increased risk of reduced semen quality leading to infertility<sup>20</sup>. Experimental observations indicated that different doses of chromium i.e. 20, 40, 60 mg/kg sodium chromate in rats caused diminution of tubular diameter, nuclear size of testicular cells and the cell population. The degree of damage is directly proportional to the dose applied<sup>21</sup>.

(iv) **Mercury**: Mercury are widely used in refinery, plastic, and paints, antiseptic, scientific instruments, photography, fuel combustion and agricultural field. This spermatoid, steroidal- and fetotoxic heavy metal and its compound mainly methyl mercury chloride exhibited structural alterations along with biochemical change, as both are androgen dependent. The control testis of albino rat of CF strain showed sharp localization of ACPase, ATPase, AMPase, AlkPase in PTM, spermatogenic cell and Leydig cell membrane<sup>22</sup>. Mercury and its compound methyl mercury chloride affected these membrane bound hydrolytic enzymes in rats resulted in sharp decrease of these enzymes, co-related with progressive degeneration of PTM. Mercury also caused the structural and functional disintegration of these enzymes due to its high affinity towards the enzyme's (-SH) groups<sup>23</sup>.

The prominent features of Mercury induced toxicity are: (1) depletion and clogging of different spermatogenic cells, (2) presence of pyknotic or karyohectic pachytene nuclei, (3) in absence of nuclear chromatin at stage XIII in dividing cells, (4) absence of noticeable lumen and (5) presence of vacuolated early elongated ST along with dispositioning of acrosome. The intensity of damage is directly proportional to the duration

of exposure<sup>22</sup>. In storage battery plant workers lead induced chromosomal aberrations are mainly the chromatin and chromosome breakage, fragments, gaps and structural abnormalities via the alteration of bases. These may be the mixed effects of other potentially toxic substances like cadmium, zinc, chromium, mercury as the workers exposed to toxic environment of more than one chemicals in the battery factory at a time<sup>28</sup>.

## MOLECULAR BASIS OF TOXICITY OF INDUSTRIAL CHEMICALS: NEW CONCEPT

**General Sperm Protein Damage :** Androgen binding protein (ABP) secreted from sertoli cells pass through the sertoli cells- lymphatic channels, bind with androgens and maintain their activity. Studies revealed that in exposed condition one of the components of ABP - heat shock protein 27 (hsp 27) plays an important role during spermatogenesis<sup>24</sup>. In exposed situation hsp 27 acted on microfilaments polymerization (*in vitro*) and microfilament dependent cell activities (*in vivo*). Welsh *et. al.* hypothesized that hsp 27 regulated the sertoli cell microfilament structure and the toxicant disrupted spermatogenesis by altering the hsp 27 regulation of sertoli cell microfilament dynamics<sup>24</sup>. Expression and localization of heat shock transcription factors : HSF1 and 2 during maturational stages of the cycle of seminiferous epithelium play a vital role in this type of study<sup>24</sup>.

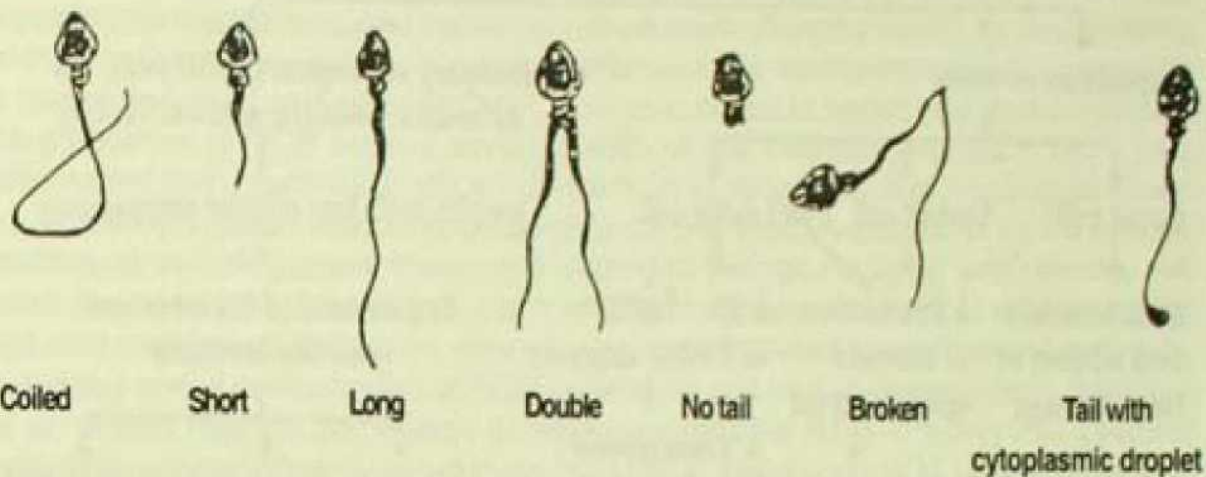
N-cadherin, desmoglein are adhesive type of protein found in sertoli cells, retained ST and in late ST, essential for maintenance of cellular adhesion of seminiferous tubules and exerted a 'signal'. The nature of the signal is unknown, but 'Inhibited Spermeation Project' indicated that there is a loss of ST-sertoli cell adhesion during spermatogenesis along with some signalling problem after exposure to pesticides heavy metals<sup>25</sup>.

Kinase type anchoring protein - AKAP 110 in human SZ and round ST binds with regulatory subunit RI1 of protein kinase A (PKA) in sperm tail in the cAMP dependent PKA pathway and RI subunit in acrosomal region of sperm head and therefore regulate both the motility and head associated functions like capacitation and acrosomal reactions. But in case of industrial workers, heavy metals disrupted the interaction of AKAP with both the isoform of PKA caused inhibition of motility and impairment of acrosome reaction leading to infertility<sup>26</sup>.

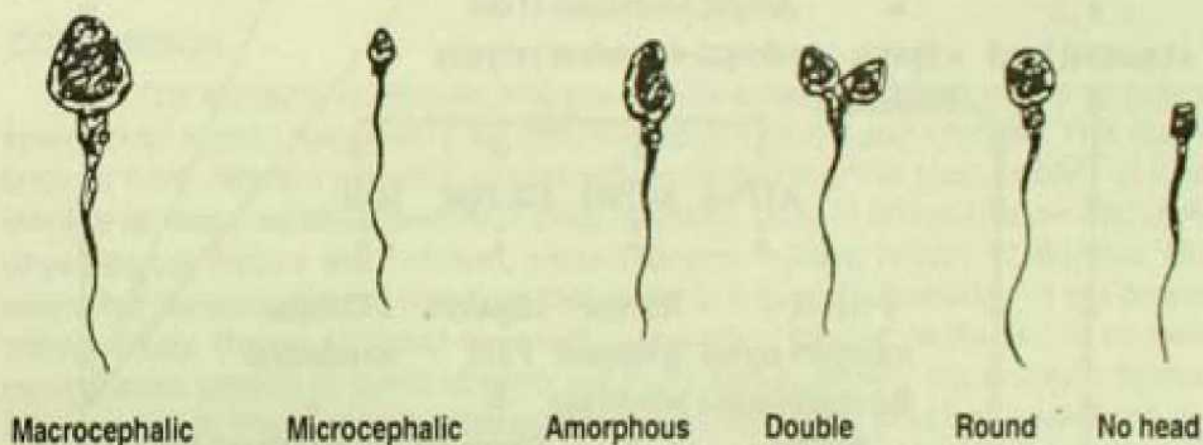
**Oxidative Damage to Sperm Head DNA and Nuclear Protein :** Histone proteins bind with DNA to give the compactness of the chromatin structure. But *in vitro* study using a synthetic metal binding motif in C terminus of histone H2A (Acetyl-TESHHK-Amine) indicated strong binding with nickel caused suppression of gene expression leading to abnormal physiological role of histone tail including locking interaction with other histones<sup>27</sup>. Nickel exposure promotes DNA damage by hydrolysis of peptide bond between glutamic acid and serine residue at pH 7 probably via auto oxidation. 8-oxo-dGTPase in normal condition prevents incorporation of promutagenic 8-oxo-dGTP into DNA. In case of industrial workers inhibition of this enzyme strongly by cadmium and copper, and weakly by nickel and cobalt caused random incorporation leading to generation of free radicals probably by electronic configurationally changes which ultimately caused oxidative DNA base damage<sup>28</sup>.

## CHEMICAL INDUCED PHYSIOLOGICAL HAZARDS ON MALE REPRODUCTION

### Tail abnormality :



### Head abnormality :



### Neck abnormality :

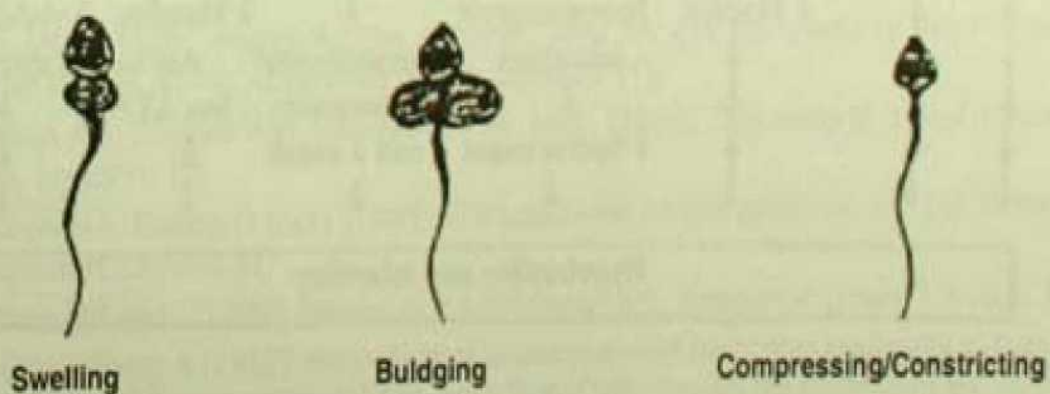


Figure 1. Industrial chemical induced teratogenicity of human sperm

# CHEMICAL INDUCED PHYSIOLOGICAL HAZARDS ON MALE REPRODUCTION

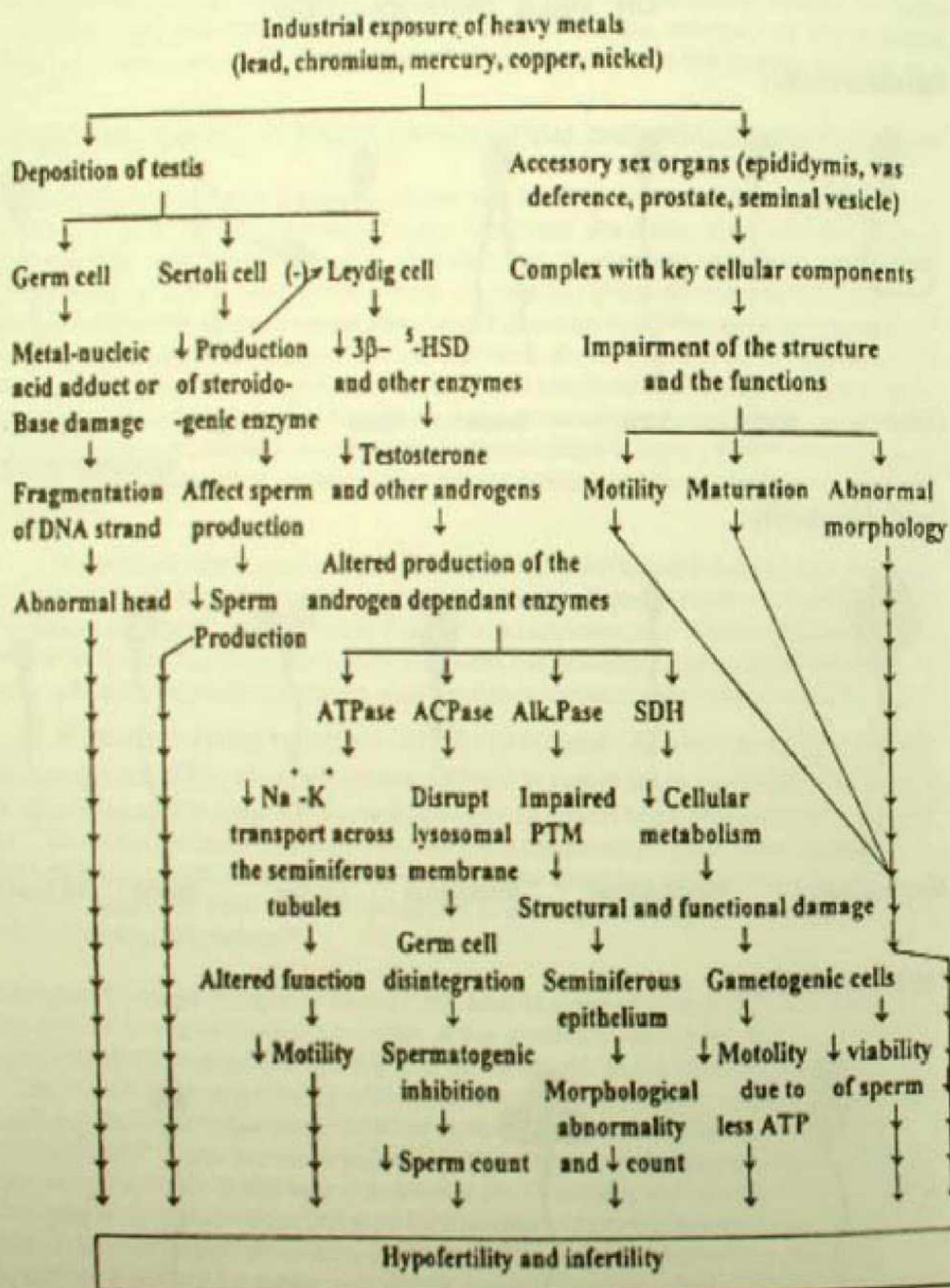


Figure 2. General view of heavy metal induced male reproductive injury

**Oxidative Damage of SZ by ROS :** Production of free radicals i.e.  $\text{OH}^\cdot$ ,  $\text{O}_2^\cdot$ ,  $\text{RO}^\cdot$ ,  $\text{CO}^\cdot$ ,  $\text{H}_2\text{O}_2$  etc. is a natural physiological phenomenon and is formed as a result of biochemical reactions by reduction of one electron from oxygen and known as reactive oxygen species (ROS) because they attack any biological compound due to excess electron in their structures<sup>29</sup>. All these free radicals produced in living cell from any physiological reactions caused impairment of oxidative phosphorylation by destabilizing coenzyme Q in the inner mitochondrial membrane or by interfering in electron transfer to that coenzyme. Lead affected Cyt P<sub>450</sub> oxidase action in testes and thus increased the production of ROS caused severe toxicity of the membrane of SZ<sup>30</sup>. ROS also affected the polyunsaturated fatty acid in membrane phospholipid of lysosomes where lysosomal hydrolases leak out to cause dystrophy and disintegration of all the cellular membrane including sperm membrane leading to 'premature aging' and ultimate cell death due to oxidative stress<sup>30</sup>. Experimentally it has been proved that environmental pollutant mainly lead, chromium, mercury and pesticide affect the rat sperm function by activating one of the pathways of ROS generation and oxidative damage<sup>30</sup>. Ichikawa *et al.* proved that the percentage of acrosome reacted SZ and acrosome reaction inducibility were significantly higher in low ROS group than in high ROS group, suggesting excessive ROS exerted a negative influence on acrosome reaction leading to impairment of fertilization and ultimately the infertility<sup>31</sup>.

## CONCLUSION

The industrial chemicals discussed so far established themselves as potent spermicidal agents responsible for disturbed sperm profile and infertility. The above findings from different scientific studies also indicated that the susceptibility of toxic insult of different metals depends on dose, duration, route of administration and other physiological factors like nutrition, socio-economic status, history of disease. But extensive literature survey explored that there is a gap of knowledge in the proper toxicity study. Hence to detect the exact cause-effect relation of chemicals on male reproductive system in terms of germ cell injury and to identify the accurate human exposure limit, the toxicity in relevant population should be monitored, because the results of these studies may be useful to warn the unforeseen male reproductive risk.

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## Molecular and Genetic Basis of Metal Resistance in Bacteria

DR. P. C. BANERJEE

**B**acterial resistances to metals/metalloids (henceforth will be mentioned as metals) as cations or oxyanions have been studied for many years. However, the overall understanding on metal resistance is rather limited with respect to the range of elements involved and the diversity of microorganisms in which such resistant systems operate. Major difficulty is that the concentrations distinguishing resistant from sensitive bacteria are not well defined because various forms of the metals, the medium components, medium pH, and culture conditions can influence metal toxicity.

Many of the heavy metals that consists of 40 elements with a density of  $>5 \text{ gm/cm}^3$ , e.g. Co, Cu, Fe, Mn, Ni, Zn etc. are essential for growth, metabolism and differentiation in very low concentrations because they provide cofactors for metalloproteins and enzymes. But the majority, e.g. Ag, Cd, Hg, Pb etc. have no indispensable biological function. At elevated concentrations, these heavy metals, both essential and non-essential, become toxic to microbes and other forms of life, because of their ability to denature proteins by blocking essential functional groups, displacing essential metal ions or modifying their active conformations. However, some bacteria are resistant to extremely high concentrations of a single metal or mixture of metals. From *Escherichia coli* to streptomycetes in every bacterial group, metal resistance is mostly plasmid-mediated encoding resistant determinants for  $\text{Ag}^+$ ,  $\text{AsO}_4^{3-}$ ,  $\text{AsO}_3^{3-}$ ,  $\text{Bi}^{3+}$ ,  $\text{Cd}^{2+}$ ,  $\text{CrO}_4^{2-}$ ,  $\text{Cu}^{2+}$ ,  $\text{Hg}^+$ ,  $\text{Ni}^{2+}$ ,  $\text{Sb}^{3+}$ ,  $\text{TeO}_3^{3-}$ ,  $\text{Pb}^{2+}$ ,  $\text{Zn}^{2+}$  and others. The related genes are also found in the chromosomes of bacteria. Plasmid-determined metal resistances have been studied to greater extent than the same of chromosomal origin because (i) plasmid-borne resistant genes usually confer higher level of resistance, and (ii) the plasmids are easy to manipulate. Like plasmid-mediated antibiotic resistances, plasmid-mediated metal resistances are highly specific. However, a general mechanism of resistance to all heavy metals does not exist in bacteria, which exhibit four basic mechanisms of resistance:

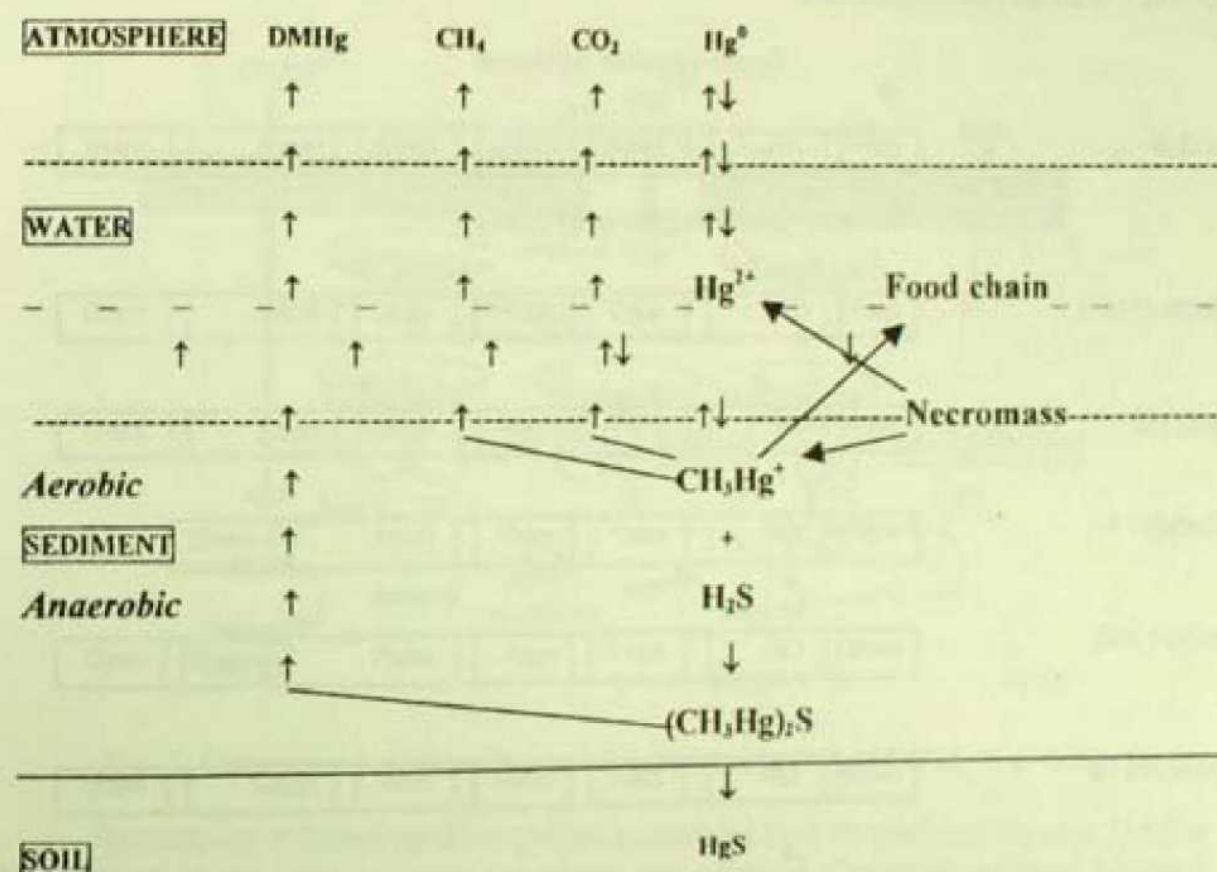
- (i) Enzymatic detoxification/ transformation of the metal ion to a less innocuous or available form.
- (ii) Highly specific active efflux pumping of the toxic ions that entered the cell by systems involved in transport of nutrient cations and oxyanions.
- (iii) Permeability barrier or reduced uptake of the toxic ions by altering the specificity of membrane transport system.
- (iv) Intra/extra-cellular sequestration by specific metal-ion binding protein or chemical.

Among these mechanisms, the second one is more common. Although efflux

pumps are the major group of resistance systems, an explanation of why instead of keeping the toxic ions out rather than to expend energy in bringing them inside the cell and then pumping them out has not been obtained. Bacterial resistance to some metals is described.

### Mercury Resistance

On average, mercury (Hg) is present at a concentration of ~ 0.5 ppm in the earth's crust, but large deposits of Hg or its ores are often found in areas of volcanic activity or tectonic plate movement. Hg is an important toxic element in the biosphere (Fig.1), which has been released in the lithosphere, atmosphere, and hydrosphere over millennia by geochemical and biological processes. But the debut of metal toxicity due to industry-related environmental pollution was reported in 1959 when poisoning of fish due to mercury was proposed as the cause of Minamata disease in humans who consumed these fishes.



**Fig. 1.** Microbial transformations in mercury cycle. The levels of Hg<sup>2+</sup> and monomethyl mercury (MMHg) are controlled by the balance of reduction and oxidation by bacteria and by the rate of bacterial methylation and demethylation. Demethylation of MMHg can be reductive (through *mer* operons) generating CH<sub>4</sub> or oxidative where CO<sub>2</sub> is produced. Both Hg<sup>2+</sup> and MMHg can pass into the food chain or be adsorbed by particulate or dissolved organic matters. Hg<sup>2+</sup> and MMHg are released back into the mercury cycle when the biomass decays. Under anoxic conditions, the reaction of MMHg and H<sub>2</sub>S (generated by sulfate-reducing bacteria from sulfates) produces dimethylmercuric sulfide. This compound is unstable and degrades to insoluble HgS and volatile dimethyl mercury (DMHg). DMHg can degrade under mild acid conditions to form CH<sub>4</sub> and Hg<sup>2+</sup> which can be transformed to Hg<sup>0</sup> (modified from Ref. 7, p. 182).

Both Gram-negative and Gram-positive bacteria exhibit mercury resistance at the level of 200 nM-100  $\mu$ M. Homologous systems are involved for mercury resistance in majority of the cases (Fig.2). It is evident that in spite of many common features, mercury resistant operons (*mer*) show diversities. Excepting the *Acidithiobacillus ferrooxidans* chromosomal and pMERPH systems, *mer* operons start with a regulatory gene *merR* which produces a unique positively acting activator protein that twists and bends operator DNA region, allowing RNA polymerase to synthesize mRNA. In *At. ferrooxidans*, two functional *merR* genes occur separately, together with functional *merC* transport genes and non-functional partial *merA* genes. The *merA* and *merB* genes code for the intracellular enzyme mercuric reductase and organomercurial lyase (breaks the C-Hg bond in the substrates), respectively. The *merD* product is a secondary regulatory protein that binds to the same DNA site as *merR* and down-regulates *mer* operon expression. The genes within *merR* and *merA* code for proteins involved in the transport of  $Hg^{2+}$  across cell membrane.

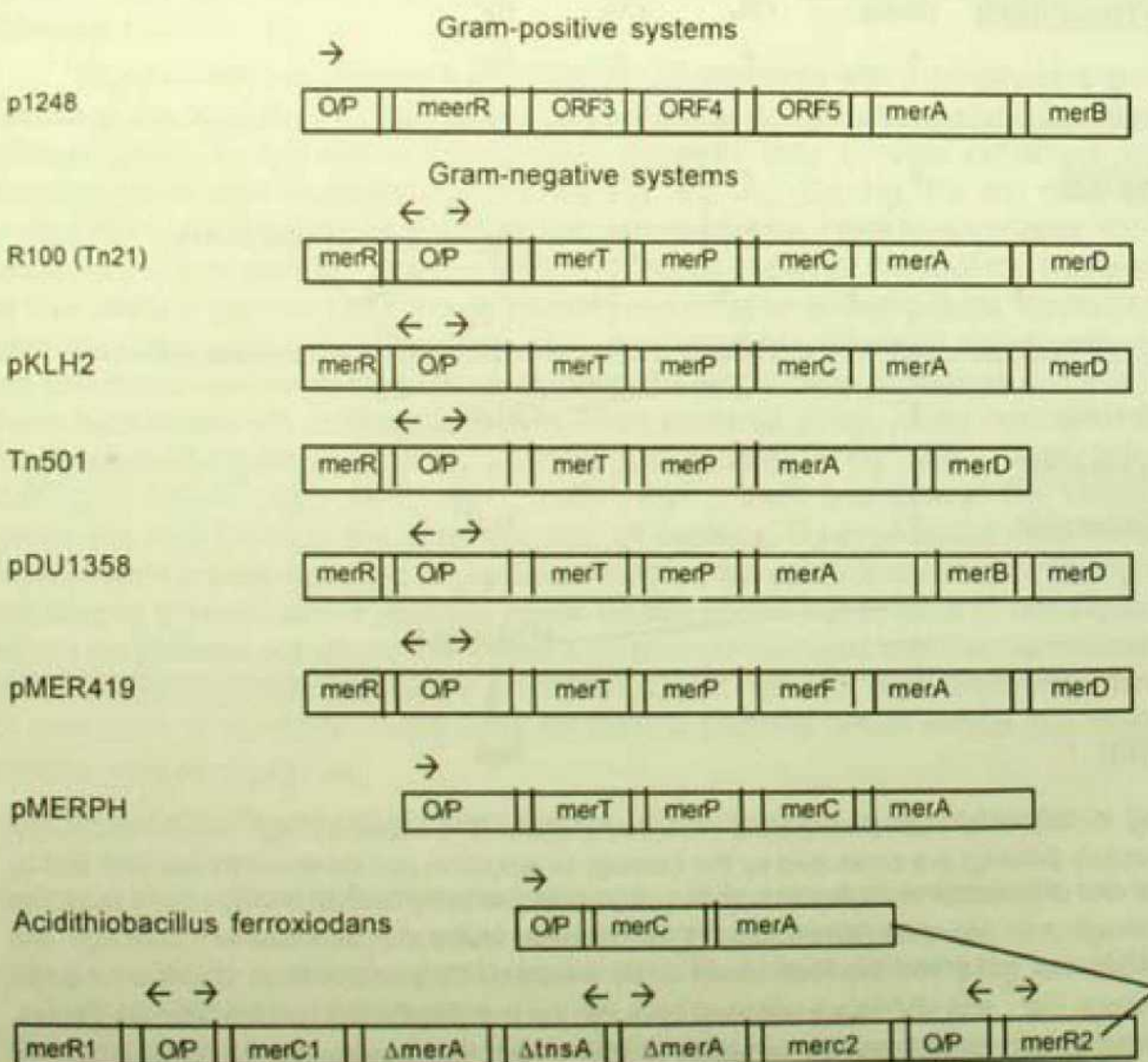


Fig. 2. Organization of genes in bacterial mercury resistance systems. Arrows indicate the directions of mRNA transcription. The line in *Acidithiobacillus* indicates a lack of linkage between the two gene clusters. O/P indicates operator-promoter region. Not drawn to scale.

Fig. 3 is a generalized model of mercury resistance in Gram-negative bacteria. It shows that  $\text{Hg}^{2+}$  in the environment [a] of a bacterial cell passes through the porins (OmpC and OmpF) in the outer membrane, where they are scavenged by the periplasmic protein MerP [b] and bind to the cysteine residues in each subunit of the protein.  $\text{Hg}^{2+}$  then passes from the cysteines in MerP to those in the transmembrane region of the inner membrane protein MerT [c]. As part of the transport mechanism,  $\text{Hg}^{2+}$  is transferred to the cysteines on the cytoplasmic face of MerT, whence they are passed [d] to the heavy metal-associated motif in the amino terminal MerP-like domain of mercuric reductase (MR) or MerA [e].  $\text{Hg}^{2+}$  is then bound at the active site and reduced to elemental mercury ( $\text{Hg}^0$ ) [f]. The volatile product is released from the enzyme and diffuses through the bacterial membranes to the environment. MMHg can diffuse in through the cell membrane, and with broad-spectrum determinants, is cleaved by organomercurial lyase (MerB).  $\text{Hg}^{2+}$  so produced is proposed to bind to glutathione in the cytoplasm and be reduced by MR.

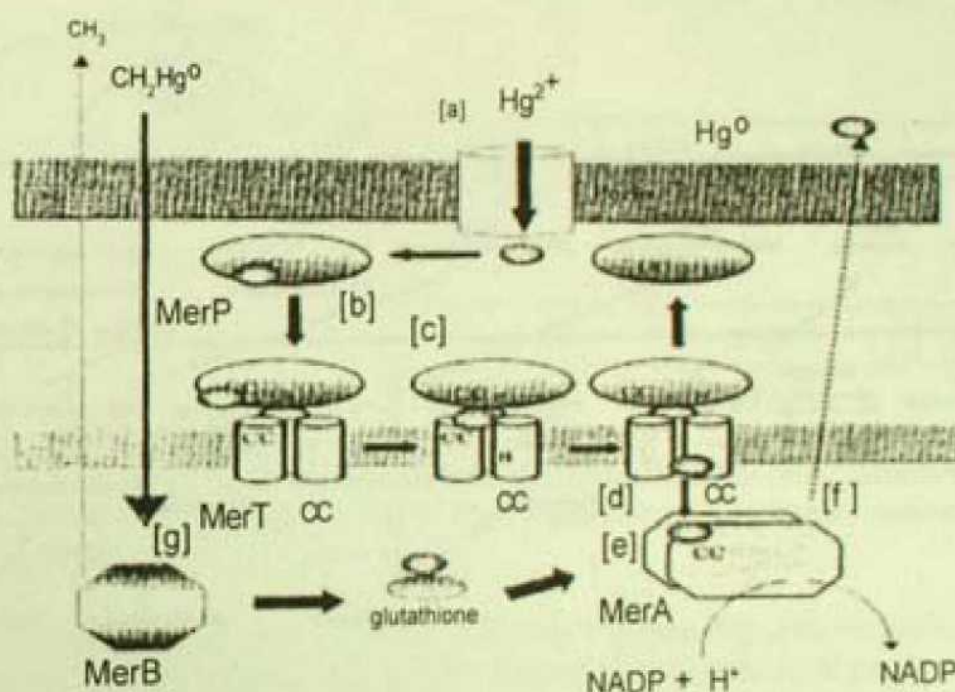


Fig. 3. Generalized model of bacterial resistance to mercury (Ref. 7, p. 185)

Resistance in Gram-positive bacteria operates by a similar mechanism, but the detailed structures of the transport proteins are different. Cysteine residues are also present in other mercury transport proteins from Gram-negative (e.g. MerC and MerF) and Gram-positive sources, and are predicted to lie in the transmembrane region. The MR from Gram-negative and Gram-positive sources are similar but differ in the number (0, 1 or 2) of MerP-like N-terminal domains and in their amino acid sequences.

### Arsenic and Antimony Resistance

Arsenic compounds, frequently present as environmental pollutants, are very toxic for most microorganisms. However, arsenic has been proposed as an essential micronutrient for humans with a predicted requirement of  $12 \mu\text{g/day}$ . Arsenic is widely spread in the upper crust of the earth, although at very low concentrations. Arsenic concentration in

soil ranges from 0.1 to more than 1000 ppm. In atmospheric dust, the range is 50-400 ppm. In seawater, the average level of arsenic may be as high as 2.6  $\mu\text{g/l}$  and in fresh water about 0.4  $\mu\text{g/l}$ . The environmental and global cycling of arsenic compounds is presented in Fig. 4. Arsenate oxyanions in water are ionized, so that at neutral pH, approx. equal amounts of  $\text{HAsO}_4^{2-}$  and  $\text{H}_2\text{AsO}_4^-$  occur. Arsenite, however, appears mostly un-ionized as  $\text{As}(\text{OH})_3$  at pH 7. Therefore, the transport substrate in and out of the cells for arsenate will be the oxyanion comparable to phosphate at approx. the same pH, whereas arsenite may move across membrane bilayers possibly unionized or be transported by a carrier protein.

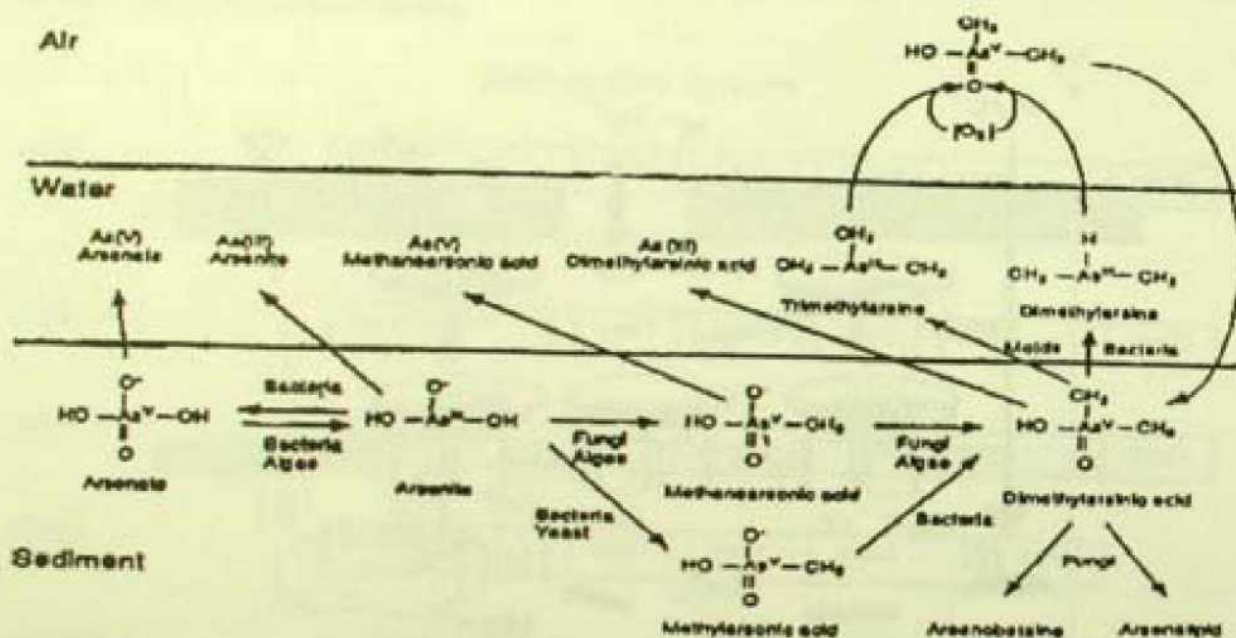
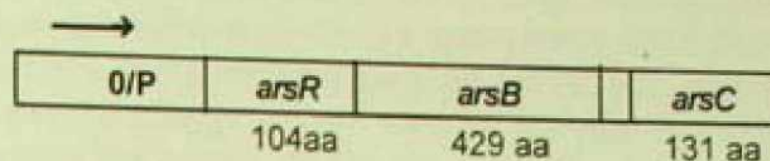


Fig. 4. Environmental and global cycle of arsenic and its compounds (reproduced from: Cervantes *et al.* FEMS Microbiol Rev. 15, 355-367, 1994).

Arsenic and antimony resistance is widely exhibited in soil bacteria. The resistance level for arsenate and arsenite varies from 12-76.9 mM and 2.8-44 mM, respectively. Gram-negative and Gram-positive bacteria both use the same energy-dependent efflux

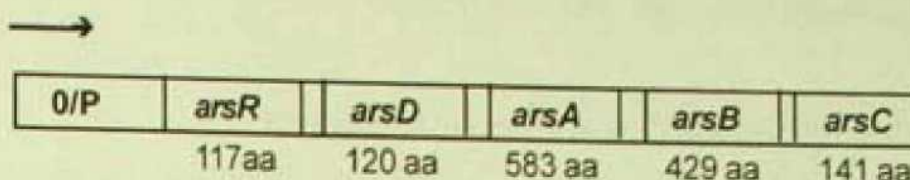
### Gram positive bacteria

*S. aureus* pl258\*

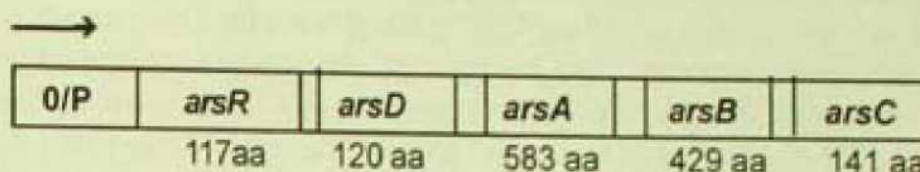


### Gram-negative bacteria

*E. coli* R773



*E. coli* R46



*A. multivorum* pKW301



*E. coli* chromosome

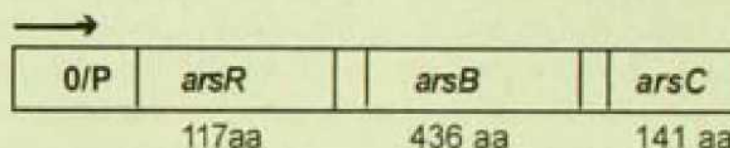


Fig. 5. Genes and sizes of predicted products for arsenic resistance in bacteria. \**S. xyloso* pSX267 has almost identical operon and encoded proteins. 0/P indicates operator-promoter region; aa, amino acids; not drawn to scale.

mechanism encoded by basically the same genes. Fig. 5 shows various arsenic resistant (*ars*) operons including the first sequenced *ars* operon of the *E. coli* plasmid R773, and *ars* operon of recently described *Acidiphilium multivorum* plasmid pKW301 - the only example of plasmid-mediated resistance in an acidophilic bacterium. The gene *arsR* codes for a transcriptional repressor protein. ArsD (product of *arsD*) acts as a second regulator and seems to act as a 'throttle' to set an upper limit on operon function. The *arsA* gene encodes a membrane-associated arsenite/antimonite stimulated ATPase that energizes the arsenite efflux pump by ATP hydrolysis. The *arsB* gene determines an inner membrane chemiosmotic (membrane-potential driven) arsenite efflux transporter protein that can function independently and as the binding site for ArsA. The gene *arsC* encodes the small soluble enzyme arsenic reductase that converts intracellular arsenate to more toxic arsenite - the ArsB substrate. ArsC activity is closely coupled with efflux of arsenite from the cells so that its intracellular concentration never increases.

In addition to the wide spread plasmid-mediated arsenic resistance, a few bacteria confer resistance to arsenite with a separate determinant that codes for enzymatic oxidation of arsenite to less toxic arsenate. Chromosomally determined arsenic

resistance generally results from effects on phosphate transport system which also transports arsenate in bacteria. Some bacteria possess two distinct phosphate transport pathways; one system takes up both phosphate and arsenate at similar rates (Pit system in *E. coli*), and the other one (Pst system) is highly specific for phosphate and transports arsenate poorly. So, natural isolates or mutants defective in or lacking the Pit pathway are usually arsenate-resistant.

### Cadmium, Cobalt, Zinc and Nickel Resistance

The levels of bacterial resistance to these metal ions were documented to be 0.05-8.7 mM for  $\text{Cd}^{2+}$ , 0.76-3.0 mM for  $\text{Co}^{2+}$ , 0.24-10.4 mM for  $\text{Zn}^{2+}$  and 0.46-3 mM for  $\text{Ni}^{2+}$  – except the acidophiles. The genetics of resistance to  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$  in *S. aureus*, *P. putida*, *At. thiooxidans*, *Ralstonia eutropha* (previous name *Alcaligenes eutrophus*), and to  $\text{Ni}^{2+}$  and  $\text{Co}^{2+}$  in *R. eutropha* has been described. The genes determining mainly efflux pumps were found mostly on plasmids. Binding factors could play a second line of defense.

At least six different operons are known to encode  $\text{Cd}^{2+}$  resistance in bacteria, three of which are present in Gram-positive bacterium *S. aureus*. The *cadA* determinant catalyzes efflux of  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$ . Some *S. aureus* plasmid (e.g. pI258) contained only the *cadA* gene while others (e.g. pII 147) contained a second resistant determinant *cadB*. The cadmium resistance encoded by *cadB* acts independent of *cadA*, is weaker compared to *cadA* -mediated resistance, and possibly is based on the synthesis of Cd-binding factor. The *cadA* gene in the *S. aureus* plasmid pI258 was studied in detail. It contains two open reading frames (ORFs) encoding a large (727 aa) and a small (122 aa) protein. The large one is essential for the  $\text{Cd}^{2+}$  resistance and was therefore referred to as *cadA* gene product. The CadA protein is a P-type ATPase that starts with a metal binding motif containing a vicinal cysteine pair (Fig. 6). This motif is similar to other Cd-, Cu- and Hg-binding regions of efflux ATPases and other proteins. A membrane ATPase region, closely homologous to other bacterial P-type ATPases, is located thereafter. The CadA protein is not sufficient to confer full resistance to Cd and Zn, the small protein (122 aa) CadC must be present. CadC represents a family of metal-binding repressor proteins, which include the ArsR and cyanobacterial metallothionein repressor SmtB. Chromosomal resistant determinant encodes  $\text{Cd}^{2+}$  efflux system, and does not encode  $\text{Zn}^{2+}$  - resistance.

The resistance to  $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Co}^{2+}$  in *R. eutropha* coded by the *czc* determinant, present in the plasmid pMOL30, involves metal dependent efflux (Fig. 7). The cations  $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Co}^{2+}$  which enter the cell via  $\text{Mg}^{2+}$  uptake system, are actively extruded from the cell, in presence of *czc*. The operon contains four ORFs designated CzcA, CzcB, CzcC, and CzcD (expressed in *E. coli*). CzcA (1064 aa), the largest protein, is essential for cation transport and is the heart of the efflux protein complex. Only CzcA has the potential transmembrane  $\alpha$ -helix, and hence is capable of forming a membrane tunnel. This protein alone probably takes part in slow  $\text{Co}^{2+}$  efflux as suggested by the residual co-resistance offered by strains carrying deletions of *czcB* and *czcC*. CzcA protein contains low amount of cysteine and histidine and hence does not offer possible

metal binding sites. On the other, the second largest protein CzcB (521 aa) of *czc* operon, contains eight histidine residues arranged in two possible metal-binding sites, and hence it is suggested to perform the cation-binding role. CzcA and CzcB function together as a  $Zn^{2+}$  efflux pump. The third protein CzcC (346 aa), is predicted to act as a modifier switching substrate specificity of the transporter from  $Zn^{2+}$  only to  $Cd^{2+}$ ,  $Zn^{2+}$ , and  $Co^{2+}$ , CzcC does not contain histidine or cysteine and hence is dependent on the CzcB protein for function. The CzcD protein (200 amino acids) is not needed for cation efflux and it is involved in the regulation of *czc*.

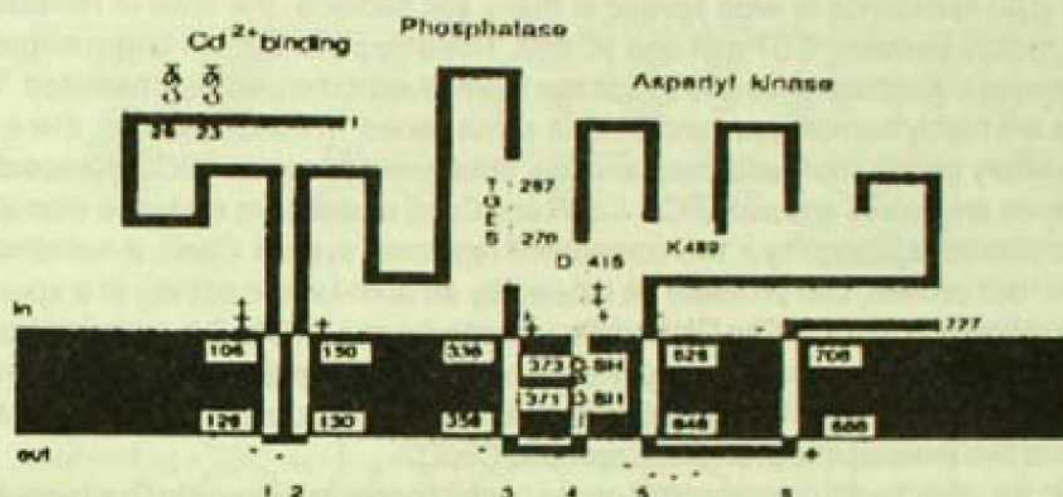


Fig. 6. The Cd-resistance ATPase of *S. aureus*. The predicted motifs ( $Cd^{2+}$ -binding, phosphatase, membrane channel and aspartyl kinase) are shown with key predicted amino acids (Ref. 5, p. 757)

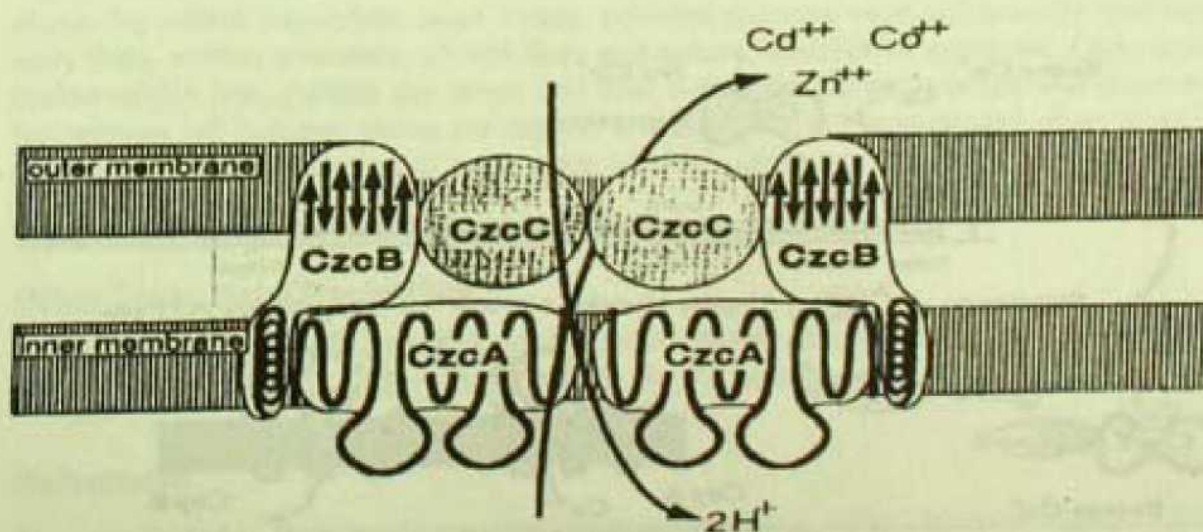


Fig. 7. Czc model for Cd, Zn and Co efflux system functioning as proton/cation antiporter consisting of inner membrane (CzcA), outer membrane (CzcC) and membrane fusion (CzcB) proteins functioning as a dimer (Ref. 5, p.761).

Resistance to  $\text{Co}^{2+}$  and  $\text{Ni}^{2+}$ , an old example of plasmid-mediated resistance, was studied in more detail in *R. eutropha*. The *cnr* system of this bacterium is encoded in the plasmid pMOL28. An energy-dependent cation-specific efflux is the mode of resistance encoded by *cnr*. DNA sequence of *czc* and *cnr* diverged significantly, but the predicted amino acid sequences show similarity in the structural genes of *cnr* CBA and *czc* CBA.

### Copper Resistance

Copper (Cu) resistance is wide spread in many soil bacteria- the level of resistance varying mostly between 0.07 mM and 20 mM. Resistance to  $\text{Cu}^{2+}$  in Gram-negative *Pseudomonas*, *Xanthomonas* and *E. coli* has been found to be plasmid-mediated. The systems are highly homologous and contain same genes. In *Pseudomonas*, there are two regulatory genes *copR* and *copS* and four structural genes *copABCD*. Respective *E. coli* genes are *pcoRS* and *pcoABCD*. CopR and CopS proteins are exclusive examples of transcriptional regulation by a 'two-component' regulatory system. CopS, a membrane-bound sensor protein, can probably be labeled by an auto-kinase activity at a specific conserved histidine residue. The DNA binding responder protein CopR is probably trans-phosphorylated on a specific aspartate residue by CopS. Structural proteins determining copper resistance are the inner membrane protein CopD, the outer membrane protein CopB, and two periplasmic proteins, CopA and CopC.

On the other hand, chromosomal genes confer copper resistance in Gram-positive pathogen *Enterococcus hirae* (previously *Streptococcus faecalis*). Two genes, *copA* and *copB* determining respectively uptake and efflux P-type ATPases, are found in a single operon (Fig.8). Chromosomal genes called *cut* affecting copper transport and resistance are present in *E. coli* and *P. syringae*. Although mechanism of resistance is not yet clearly established, preliminary evidence for copper efflux has been found in the *E. coli* system.

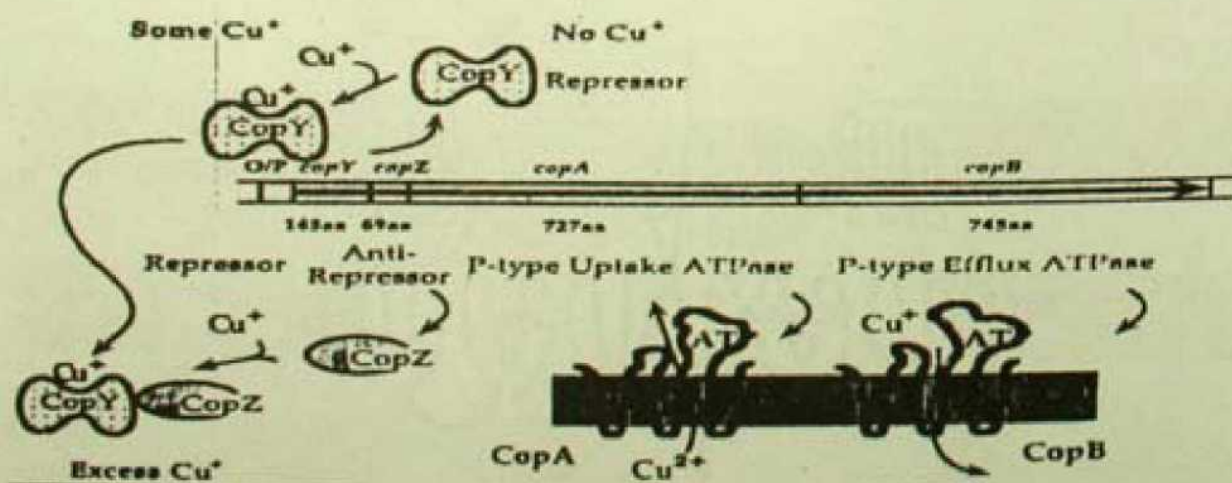
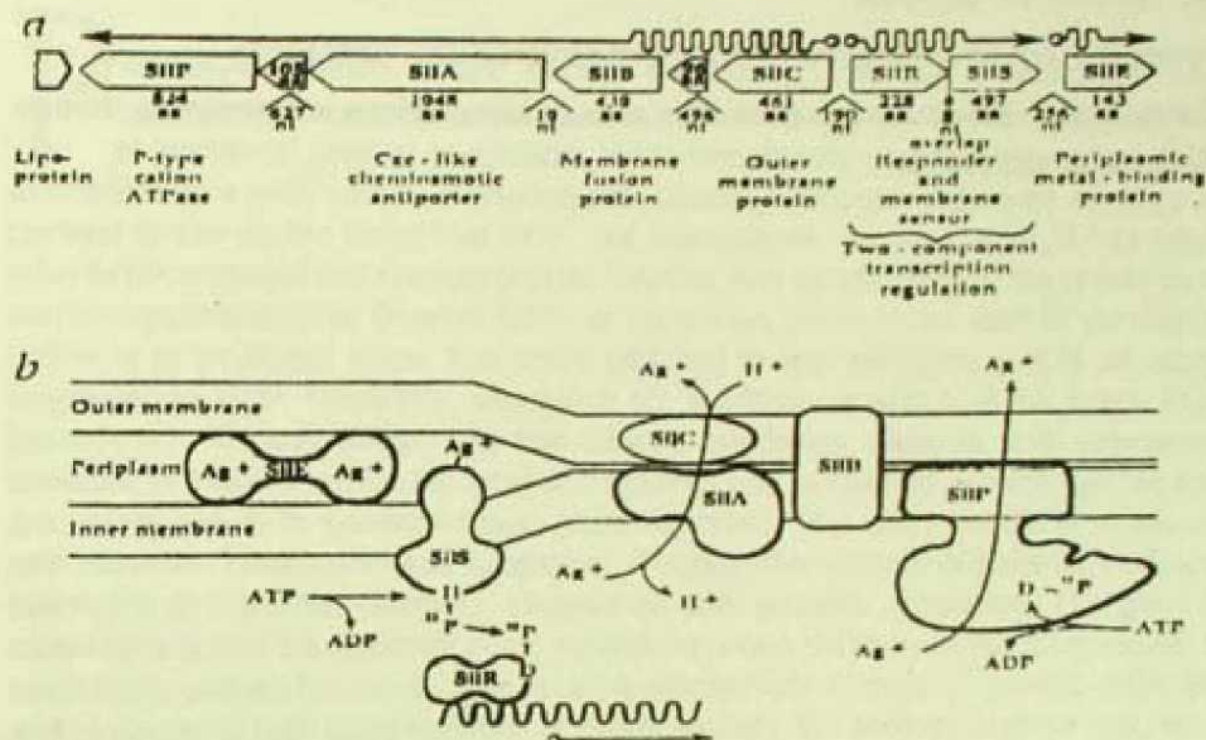


Fig.8. Transport and regulation of copper in *E. hirae*. The *copA* and *copB* determine copper uptake and efflux P-type ATPases, respectively. The ATPases are shown passing six times across the membrane (Ref. 5, p.763).

## Silver Resistance

The silver resistance determinant from a *Salmonella* (a hospital burn ward isolate) plasmid pMG101 contains nine open reading frames, arranged in three measured and divergently transcribed RNAs (Fig. 9). The resistance operon encodes a silver-specific periplasmic protein (SilE) plus apparently two efflux pumps: (i) a P-type ATPase (SilP), and (ii) a membrane potential-dependent cation/proton antiporter (SilBCA). The *sil* determinant is regulated by a two-component system consisting of a membrane sensor (*silS*) and a transcriptional responder (*silR*), which are co-transcribed.



**Fig. 9.** Silver resistance-determinant, genes, transcripts and protein products. **a.** Top line shows the mRNA transcripts: open circles, potential promoter regions/transcript start sites; wavy lines, mRNA synthesis; straight lines and arrows, direction of synthesis. Open boxes (below mRNA line) indicate the genes and their transcriptional and translational directions. Nucleotides (nt) between genes are marked and sizes of gene products are given in amino acids. **b.** Proposed function of each gene product, deduced from homologies to known proteins.

## Other Toxic Metal Resistance

In addition to the specific resistances discussed above, resistances to several additional metal ions, e.g.  $\text{CrO}_4^{2-}$ ,  $\text{TeO}_3^{2-}$ , ions of Bi, B, Pb, Ti, and Sn were reported.

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## Premature Ovarian Failure : A Look into its Pathogenesis through Animal Models.

DR. SYED N. KABIR

**P**remature ovarian failure (POF), a condition causing amenorrhea and hypergonadotropic hypoenestrogenism before the age of 40 years, affects 1% of women in general population. In chromosomally competent women, POF demonstrates a wide variety of phenotypes, possibly because of diverse etiology. In contrast to our earlier belief that POF, like menopause, is irreversible, it has been recently documented that residual ovarian function may remain despite the presence of elevated gonadotropins. Ovarian follicular apparatus, either in the form of primordial follicle or at an atretic stage, has been identified in approximately ~ 50% of cases diagnosed as POF. Therefore, depending on the follicular status of the ovary, POF patients may be sub-divided into two distinct categories: patients, with premature depletion of follicles (afollicular type) and patients with remaining ovarian follicles that are unresponsive to gonadotropins (follicular type). The latter condition is usually referred to as "Irresistant ovary syndrome". A substantial number of patients, perhaps, belonging to the latter category, experience spontaneous remission. The general consensus is that the resistant ovary syndrome is one pathway in the progression to irreversible ovarian failure regardless of the mechanism. Except for genetic disorders and exposure to high dose radiation or chemotherapy, the etiology and the regulatory mechanisms underlying pathogenesis of POF are largely unknown, and oocyte donation as yet remains the only evidence-based effective line of therapy for the management of POF.

We have been working for the past few years to explore the possible pathophysiological attributes of POF. A number of reports document that women with galacto-saemia eventually develop POF. While some patients clearly demonstrate ovarian failure related to follicle depletion, others might exhibit normal appearing primordial follicles in ovarian biopsy but without growth and development. The pathophysiological attributes of POF in galactosaemic and other chromosomally competent women are likely to be different but in both cases the disease possibly progresses through similar clinical pathways. So, investigation on galactosaemic subjects may provide clues to some natural mechanisms underlying the pathogenesis of POF. Administration of galactose to rats during pregnancy produces a sequel of abnormalities in the litters which are characteristic features of human galactosaemia. Thus the rodents provide an excellent model system for galactose toxicity, which is easy to acquire, maintain and modulate. Therefore, we explored the suitability of

experimentally induced galactose toxicity in rat as a model to study the underlying mechanism of POF. We demonstrated that prenatal and early neonatal exposure to high concentration of galactose produced a galactosemia-like condition with the accumulation of galactose and galactose-1-phosphate, and led to delayed onset of puberty. The animals developed hypergonadotropic hypooestrogenism and follicular refractoriness to gonadotropins, the two major diagnostic features of POF. Histopathological study showed ovarian follicular deficiency in many rats, while others exhibited normal complement of follicles, though they were unresponsive to exogenous gonadotropins. At the same time, the ovaries represented marked preponderance of atretic follicles. Deficiency of follicular complement might be attributed to either an accelerated atresia of normal complement of follicles or a reduced number of primordial follicles in the initial pool resulting from migratory failure of germ cells. Interestingly, we observed adversely affected germ cell migration as evidenced by the presence of comparatively lower number of germ cells in 12-15 day old experimental embryos at day-specific sites on the migratory path. Thus, it has been suggested that deficient follicular reserve ensued as a consequence of dually executed toxic effects of galactose. At one end, galactose antipathetically affected germ cell migration leading to the development of ovary with scarce initial pool of germ cells, while on the other hand it accelerated the rate of follicular atresia. Both effects converged together and attributed to premature depletion of ovarian follicular reserve with consequent development of hypergonadotropic hypooestrogenism.

Another intriguing aspect of galactose toxicity was a significant attenuation of ovarian response to gonadotrophin in the face of apparently normal pool of follicular reserve. Of special interest was to observe that the follicular refractoriness to exogenous gonadotropins significantly subsided as the endogenous gonadotropins were suppressed by chronic administration of GnRH-agonist. This differential follicular response with and without pituitary desensitization as well as attenuated rate of FSH-stimulated estradiol production by granulosa cells *in vitro* by the experimental sera, led us to hypothesize that the endogenous gonadotropins produced under galactosaemia perhaps exerted some antigonadotropic effects.

Gonadotropins are the members of glycoprotein family of hormones. Carbohydrates attached to the protein core of these hormones influence a number of intra-cellular and extracellular processes including activation of the respective receptors efficient triggering of signal transduction. As with other glycoprotein hormones, FSH is produced and released as an array of isoforms that differ from each other in respect of their oligosaccharide attachments. Defective glycosylation may transform an agonistic hormone into one of antagonistic nature.

Galactose represents an important constituent of the carbohydrate side-chain of FSH molecule. Under these circumstances of aberrant galactose metabolism, we detected structurally altered isoforms of FSH from experimental sera through isoelectric focussing and immunoblotting. Consequently, as opposed to the acidic forms of bioactive FSH, galactose-deficient neutral isoforms of FSH are produced, possibly due to deficient incorporation of sialic acid. These neutral isoforms of FSH retain their capacity to bind with their receptors, but fails to induce signal transduction, and therefore act as anti-FSH. Subsequently, we tested the acceptor capacity of serum

proteins to incorporate galactose in the presence of UDP ( $^3\text{H}$ ) galactose as donor and a purified galactosyl transferase. Our results indicated greater incorporation in our experimental model suggesting a deficiency of galactose in the carbohydrate moiety of gonadotropins to which sialic acid was linked. These experiments further confirmed the production of biologically inactive deglycosylated forms of gonadotropins (particularly FSH), which attributed to the ovarian refractoriness of gonadotropins under conditions of experimental galactose toxicity.

Further investigation showed that in addition to increased levels of galactose-1-phosphate and deficiency of UDP-galactose, the aberrant galactose metabolism under galactose toxicity was characterized by attenuated activity of galactosyltransferase (GalTase), the enzyme that catalyzes transfer of galactose from UDP-galactose to terminal N-Acetylglucosamine in the process of synthesis of carbohydrate component of the glycoproteins.

In addition to its traditional location in the Golgi complex, a subpopulation of GalTase is also known to exist on cell surface, which act as receptor for glycoconjugates in the extracellular milieu and plays important roles in many cellular processes including their locomotion. PGC are known to possess surface GalTase and extracellular matrix on the path of PGC migration has been documented to possess laminin, a well-known substrate for GalTase. We therefore investigated if attenuated GalTase activity was the missing link between galactose toxicity and inhibited oogonial migration. Embryonic GalTase activity was modified by different modifier principles and its impact on oogonial migration was evaluated. We observed that perturbation of GalTase either by altering substrate specificity or by inducing competitive substrate inhibition led to significant inhibition of germ cell locomotion. Induction of premature catalysis of GalTase- substrate reaction by exogenous UDP-galactose, however, had no influence on oogonial locomotion. We therefore provide strong supportive evidence in favour of an enzymatic role played by GalTase in the process of germ cell migration.

## Bioactivity of the Phytochemicals

DR. BRATATI DE

An enormous variety of organic substances are elaborated and accumulated by plants. Excluding the primary processes of sugar and protein biosynthesis, there are three main routes to the wealth of these chemical compounds found in plants – the acetate malonate, acetate mevalonate and shikimic acid pathways, which are interrelated. These pathways are ubiquitous and the first products formed in quantity can be considered primary metabolites and their derivatives being secondary metabolites. Variations in primary processes also lead to secondary metabolites. Thus the pentose phosphate pathway producing the primary metabolites glucose and fructose also generates the rare sugars found in plants, while variations in the pathways synthesizing the protein amino acids give the non-protein amino acids. Plants have long been exploited because of the presence of these phytochemicals. Not only do plants provide the carbohydrates, proteins and fats necessary to the diet of man and other animals, but they also provide most of the essential vitamins. For thousands of years plants have also been used as medicines.

Atropine and hyoscine (from *Atropa sp.*, *Datura sp.*, etc.), physostigmine (from *Physostigma venenosum*), pilocarpine (from *Pilocarpus microphyllus*) are the plant alkaloids used in ophthalmic preparations. Atropine and hyoscine are used to a large extent in ophthalmic practices to dilate pupil of the eye. Physostigmine salicylate is used for contracting the pupil of the eye, often to combat the effect of mydriatics. Salts of pilocarpine are used in ophthalmic practice, as they cause contraction of the pupil of the eye. In early glaucoma treatment they serve to increase the irrigation of the eye and relieve pressure.

The hallucinogenic property of lysergic acid diethyl amide derivative (LSD) is well known. The lysergic acid forms the nonpeptide portion of a number of ergot (the sclerotium of a fungus, *Claviceps purpurea*, arising in the ovary of the rye, *Secale cereale*) alkaloids. Three main types of narcotic products are produced from *Cannabis sativa*. These are ganja, bhang or hashish. In America and Europe the product used by addicts is known as marihuana. The narcotic resin consists of over 60 compounds (cannabinoids). Some principal components are cannabinol, tetrahydrocannabinol (THC), cannabidiol, cannabigerol, cannabichromene. D<sup>9</sup>-THC is the principal psychoactive constituent. Cocaine (obtained from *Erythroxylum coca*) was one of the earliest drugs used as a mental stimulant. Cocaine and its salts were the earliest of the modern local anaesthetics, but, because of their toxic and addictive properties, their use is now almost entirely confined to ophthalmic, ear, nose and throat surgery. Caffeine,

theobromine, theophylline which are the constituents of beverages coffee, tea, coca, kola stimulate mental activity. The current popularity of St John's wort (*Hypericum perforatum*) relates to its unregulated availability for the treatment of mild to moderate depression. In the USA, for the first eight months of 1999, it ranked second to *Ginkgo* as the best selling product of the herbal mainstream market. Until recently the anthraquinone hypericins were regarded as the sole antidepressant principles of *Hypericum*.

Morphine is effective for the relief of severe pain. Ephedrine (from *Ephedra sp.*), theophylline are bronchodilators. Ipecac, liquorice, squill, lobelia are expectorant drugs. Preparations of ergot alkaloids were traditionally used in child birth and then largely replaced by the isolated alkaloid ergometrine.

A number of plants are used as laxatives and purgatives. The husks of seeds of *Plantago ovata* (Ispaghula Husk) are used in the treatment of chronic constipation. Hydrophilic colloids function as bulk producing laxatives. An indigestible vegetable fibre also absorbs water and provides bulk. Dried leaflets of *Cassia senna* (Alexandrian senna) and *C. angustifolia* (Tinnevely senna) constitute a useful purgative for either habitual constipation or occasional use. Two active glycosides are sennoside A and sennoside B. Castor oil is a fixed oil obtained from the seeds of *Ricinus communis* (Euphorbiaceae). Castor oil, once widely used as a domestic purgative, is now more restricted to hospital use for administration after food poisoning. The purgative action of the oil is said to be due to free ricinoleic acid and its stereoisomer, which are produced by hydrolysis in the duodenum. Other drastic purgative is jalap resin (obtained from the roots of *Ipomoea purga*), now little used for this purpose.

Many volatile oils are used as carminatives. Dill (fruits of *Anethum graveolens*) oil is used for the relief of flatulence, especially in babies. Other plants or oils used as carminatives include caraway (fruits of *Carum carvi*), fennel (fruits of *Foeniculum vulgare*), peppermint (leaves of *Mentha piperita*), thyme (leaves and flowers of *Thymus vulgaris*), nutmeg (kernels of the seeds of *Myristica fragrans*), ginger (rhizome of *Zingiber officinale*), clove (flower buds of *Syzygium aromaticum*), cinnamon (shoot bark of *Cinnamomum zeylanicum*). Oil of eucalyptus, distilled from the fresh leaves of various species of *Eucalyptus*, is much used for alleviating the symptoms of nasopharyngeal infections, for treating coughs and as decongestant.

Liquorice, the dried roots of *Glycyrrhiza gabra* (Leguminosae), has long been employed as demulcent and mild expectorant. The recognition of deoxycorticosterone effects of liquorice extracts and glycyrrhetic acid (a triterpenoid) has led to its use for the treatment of rheumatoid arthritis and various inflammatory conditions. The flavonoid component of the root exerts spasmolytic and antiulcerogenic activity. Liquorice may give symptomatic relief from peptic ulcer pain.

Quinine, isolated from the bark of various species of *Cinchona*, constituted the most effective agent for the treatment of malaria. But there was a decline in the use of quinine due to the development of synthetic antimalarials. However, development of *Plasmodium falciparum* resistant to chloroquine and other antimalarials led to resurgence in the use of quinine which is effective against chloroquine-resistant organisms. Artemisinin, an unusual sesquiterpene lactone from *Artemisia annua* is effective against

chloroquine resistant strains of *Plasmodium vivax* and *P. falciparum* as well as against cerebral malaria. Emetine, the alkaloid of ipecac (*Cephaelis ipecacuanha*) is another antiprotozoal drug used in the treatment of amoebic dysentery. The alkaloid is highly active against *Entamoeba histolytica* *in vitro*. Oil of *Chenopodium* has been extensively used in hookworm disease.

In the circulatory system thrombi may be caused on the arterial side as a result of the adhesion of blood platelets to one another and to the walls of the vessels. This platelet aggregation is triggered by the platelet activating factor which is released from activated basophils. Five diterpene lactones Ginkgolides A, B, C, J, M are platelet-activating factor (PAF) antagonists. Preparations of *Coleus forskohlii* have long been used in Ayurvedic traditional medicine particularly for the treatment of heart diseases, abdominal colic etc. Forskolin (a diterpene) obtained from the roots of this plant has hypotensive, spasmolytic, cardiostimulant and platelet aggregation inhibitory activity. Cardiac glycosides of *Digitalis sp.* are used for the treatment of failing hearts. The cardiac glycoside thevetin obtained from the seeds of *Thevetia peruviana* has found use in continental Europe and is particularly useful in cases of mild myocardial insufficiency and where digitalis intolerance exists. The cardiac glycosides can be used to control atrial cardiac arrhythmias. There are a number of other drugs such as quinidine (obtained from barks of several *Cinchona sp.*) which act on both supraventricular and ventricular arrhythmias. Of the hypotensive plant drugs *Rauwolfia* and its alkaloid reserpine were recognized in allopathic medicine in the early 1950s. In recent years importance has been given to the association of high levels of blood cholesterol and plasma triglycerides with atherosclerosis and ischaemic heart disease. Published data supports the hypothesis that garlic lowers serum total cholesterol and improves the lipid profile.

Plant materials have been used in the treatment of malignant diseases for centuries. The most successful of higher plant materials used in cancer chemotherapy are the alkaloids of *Catharanthus roseus*. Vincalcalcin (vinblastine) and leurocristine (vincristine), the two dimeric indole alkaloids, are now extracted commercially from the aerial parts of this plant and used either alone, or in combination with other forms of therapy for cancer treatment. Vinblastine is mainly useful in the treatment of Hodgkin's disease, a cancer affecting the lymph glands, spleen and liver. Vincristine is clinically more important than vinblastine, and is especially used in the treatment of acute lymphocytic leukaemia in children. It has other applications for lymphomas, small cell lung cancer, cervical and breast cancers. The vinblastine and vincristine content in the plant is very low. Over 500 kg of the plant is needed for the production of 1 g of vincristine. *Podophyllum* resin has a cytotoxic action. Etoposide is a lignan derivative obtained semisynthetically from podophyllotoxin and is used in the treatment of small cell lung cancer and testicular cancer as well as lymphomas and leukaemias. Taxol (paclitaxel) is isolated from various *Taxus* species. It is used clinically in the treatment of ovarian cancers and non-small cell lung cancer. The content of paclitaxel is also very low (0.01-0.04 % of the dried inner bark) and production of 1 kg of paclitaxel requires 9000 kg of bark from 2000-3000 trees. Taxotere is a side chain analogue of taxol, which has also been produced by semisynthesis from 10-deacetylbaicatin III, a constituent of *Taxus*. It has improved water solubility and is used in the treatment of breast cancers.

The liver which is the principal organ of metabolism and excretion, is subjected

to a number of diseases. The most widely used hepatoprotective agent is silymarin, a purified extract from the fruit of *Silybum marianum*. In Indian medicine many plants are so used. The leaf juice of *Andrographis paniculata* is a household remedy for many ailments of the alimentary tract. It is one of the most active ingredients of the Indian polyherbal preparations used to treat liver ailments. The active antihepatotoxic principle is probably the diterpene lactone andrographolide.

A number of promising plant constituents with anti-HIV activity have been discovered. Castanospermine, an alkaloid isolated from the seeds of *Castanospermum australe* (Leguminosae) was tested against HIV on the basis that this compound inhibits  $\alpha$ -glucosidase I and II, which control the formation of glycoproteins in the viral coat, then, without the essential envelope structure the virus would be unable to infect healthy white blood cells. The antiviral test proved positive and various O-acyl derivatives have since been shown to be upto 20 times more active than castanospermine itself. Although the toxicity levels are unsatisfactory for clinical use it constitutes a prime lead compound. Several species of a genus of gourds (*Trichosanthes*) contain a toxic protein trichosanthin. Preparations based on this compound appear to have ribosome-inactivating properties and selectively kill cells infected with HIV.

Plant secondary metabolites are also important as precursors for the synthesis of some drugs, particularly hormones and steroids. Diosgenin (from *Dioscorea sp.*), solasodine (from *Solanum sp.*) and other sterols are used as the starting material for the synthesis of steroidal drugs.

Phyto-oestrogens are non-steroidal plant substances of flavonoid constitution exhibiting oestrogenic properties. They have recently received considerable attention. Both structurally and functionally they are similar to oestradiol and related sex hormones and exert weak oestrogenic effects. Epidemiologic data illustrate strong associations between diets high in foods containing phytoestrogens and a reduction in coronary heart disease, cancers and menopausal symptoms. In the plant they occur free or in glycosidic form, in the latter case being hydrolysed by colonic bacteria to give the active aglycone. Genistein and daidzein are the principal examples.

The oxygen molecule is very stable in ground state, but it is changed into  $O_2^{\cdot-}$  (superoxide radical),  $H_2O_2$  (hydrogen peroxide),  $OH$  (hydroxyl radical) by environmental pollutants, radiolysis, UV and reduction pathway to  $H_2O$  in living tissues. These oxygen radicals induce some oxidative damage to biomolecules like carbohydrates, proteins, lipids, DNA thus accelerating aging and illness. Among these oxygen radicals  $OH$  is the most reactive and induces severe damage to the adjacent biomolecules. The harmful action of the free radicals can however be blocked by antioxidant substances which scavenge the free radicals and detoxify the organism. Current research into free radicals has confirmed that food, rich in antioxidants, play an essential role in the prevention of cardiovascular diseases and cancers and neurodegenerative diseases including Parkinson's and Alzheimer's diseases as well as inflammation and problems caused by cell and cutaneous aging. Antioxidant nutrients vitamin E, vitamin C and  $\beta$ -carotene may play a beneficial role in the prevention of several chronic disorders. Flavonoids, tannins, anthocyanins and other phenolic constituents present in food of plant origin are potentially antioxidants.

Besides their medicinal value, plant poisons have been used for thousands of years to kill animals. Arrow poisons are generally prepared from plants containing cardiac glycosides or alkaloids. The term 'curare' is a generic one applied to various South American arrow poisons. These extracts are made from a number of different plants, particularly members of the Menispermaceae (e.g. *Condrodendron sp.*) and the Loganiaceae (*Strychnos toxifera*, *S. guianensis* etc.). Menispermaceous curare contain tubocurarine. Curare is now little used except as a source of alkaloids. Tubocurarine chloride is used to secure muscular relaxation in surgical operations and in certain neurological conditions.

Many plants contain insecticidal compounds. Some of them are used on large scale. These include nicotine from *Nicotiana* species, rotenone from *Derris* and *Lonchocarpus* species, pyrethrins from pyrethrum (*Chrysanthemum cinerariaefolium*), azadirachtin from neem (*Azadirachta indica*).

Undoubtedly, the plant kingdom still holds many species of plants containing important bioactive phytochemicals which have yet to be discovered. Large number of plants are constantly being screened for their possible pharmacological value.

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# **Beneficial Effects of Tea in Human Health and Disease**

**DR. LALIMA CHAUDHURY**

**D**iagnosis of and therapy for major diseases are usually high cost elements in health management. Thus there has been an increasing emphasis on research to develop an understanding of the causes of diseases as well as the action modulating factors, as a basis for prevention.

Herbs, spices and condiments are part of diet worldwide. Many of them have known pharmacological and therapeutic properties relevant to human health and most contain potentially bio-active compounds. General knowledge of the therapeutic qualities of many herbs and some spices suggests that further scientific evaluation may generate evidences of their therapeutic usefulness in the treatment of many chronic diseases. This is particularly true for tea, tea roots and their constituents.

Tea is the most commonly consumed drink in the world, after water. The areas where the most tea is consumed include India, China, Russia, Turkey, UK, North Africa, Japan, USA and Indonesia. UK has the highest per capita tea consumption of any country. Worldwide, black tea is the type most commonly consumed, green tea is more commonly drunk in Japan, China and Taiwan.

Tea leaves have been used in health care products for thousand of years. But in recent past, large number of studies have been undertaken to explore the beneficial effect of tea in health and diseases.

There are varieties of tea available across the world, green tea, black tea and oolong tea. Green tea is typically consumed in East Asian countries and is prepared by processing fresh tea leaves rapidly to prevent any fermentation.

Black tea is the predominant category of tea available worldwide and is consumed typically in the USA, Europe, Africa, and India. It is made by crushing and drying fresh tea leaves to effect fermentation, during which some of the tea catechins combine to form complex theaflavins and other flavonoids, which are beneficial to overall human health.

Green tea is typically consumed in East Asian countries and is prepared by processing fresh tea leaves rapidly to prevent any fermentation.

Oolong tea is a partially fermented tea product and has a very different flavour and chemistry. This category of tea is manufactured and consumed in China and Taiwan.

Research has now been established that our morning and afternoon cuppa is actually good for health. Anytime in between and later in the day too wont cause harm.

So the renewed interest in tea in the world is because some properties of this beverage has a direct positive effect on busy lives.

So detailed focussed research on the health benefits of tea is of recent vintage. Initially, such research involved green tea containing high amount of free catechins and low quality of catechin oxides. Scientific interest in search of medicinal properties of black tea containing higher quantity of catechin oxides generally termed as thearubigin and theaflavins and lower quantity of free catechins has taken birth only recently. (1-4).

Epidemiological studies suggest a protective effect of both green and black tea against various human cancers, including those of colon and rectum (5).

In the human stomach:

- Tea catechins act on *Helicobacter pylori*, which causes digestive gastritis (6).
- Daily administration of tea catechins appear to exert a positive effect on the intestinal flora of humans as well as animals (7).
- It has been reported that green tea polyphenol improved bowel movement regularity in people having tea catechins in tablet (7).
- Stool consistency was also softened with black tea in people having tea daily in a four week trial (8). They excluded any involvement of caeffine, milk or sugar and ascertained that the effect was due to blak tea polyphenol.

In another study, it has been reported that tea, independent of its caeffine content, did not induce gastro-esophageal reflux (9).

Accumulating all the recent evidences, it can be said that tea has a definite positive influence on the digestive tract.

Tea extract contain so many constituents that the single or synergistic effects of other constituents cannot be altogether ruled out.

Therefore, it is necessary to evaluate various biological and pharmacological tests of tea extract in general, in an integrated way.

There has been some extensive studies on anticancer, antidiabetic, antihypertensive, antioxidant activity of green tea polyphenols (10).

Black tea and its constituents have been reported to posses prokinetic, anti-ulcer, antihyperglycemic (Chaudhury et.al, Maity et.al, Gomes et.al.), antidiarrhoeal and antibacterial activities. (work is in progress).

We have reported that tea root extract showed some excellent activity against Ehrlich ascites carcinoma ( EAC ) in Balb-C mice indicating the possibility of development of novel anticancer drug.

In addition, our group has also done some experimental studies of black tea and its constituents on rat phrenic nerve diaphragm preparation.

All references of our work are given in R & D achievements.

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# Dust Filtering Property of Avenue Trees and the Effect of Air Borne Particulate Matters on Growth and Yield of Paddy Plants

PROF. T. M. DAS.

For the Hyde Park, a green area of only one square mile in size in the centre of London, an average reduction in the smoke concentration of 27 per cent was found (9). It is important to note that over a distance of less than 200 yards the vegetation is able to reduce the concentration of suspended particulates by about  $120 \mu\text{g}/\text{m}^3$ . Brandt and Heck (3), Landau and Brandt (7), Heggestad and Heck (5), Hill (6), Autonovics, Bradshaw and Turner (1), and Reilly (10) studied different aspects of the effect of air pollutants on crop plants and avenue trees. An interesting study in the Soviet Union (2) was set up to find out which tree genera exert the greatest filter effects. For this purpose the dust per unit area of leaf surface was weighed. The best vegetative dust filters in descending order and in  $\text{g}/\text{m}^2$  of leaf surface were lilac with 2.33; maple with 1.11; linden with 0.61; and poplar with 0.26. It was calculated that 400 poplars which are the poorest dust collectors, spread over 2.5 acres, would filter out 0.375 tons of dust during leaf-bearing season.

## MATERIALS AND METHODS

For the study of dust filtering capacity of different avenue trees : leaves of different species of trees growing in the same area of the Indian Botanic Garden, Howrah were carefully collected from approximately of the same height from the ground level and deposition of dust per unit area of the leaf surface was measured by removing the dust particles from the upper as well as lower surface of the leaf with the help of very clean camel hair brushes and weighing them with chemical balance (electrical).

The effect of particulate matters on crop plant was studied at the Calcutta University Experimental Farm at Baruipur. A clean glass vessel previously weighed in an electric balance was kept at the level of 30 cm height from the ground level in meteorological data collection centre of the farm. After seven days the vessel was weighed again and the collection was subjected to microscopic examination and chemical analysis. Dust collected in a larger vessel also was sprinkled on the leaves of boro paddy var. Ratna at the rate of  $1 \text{ kg}/3\text{sq m}/\text{week}$ . These rice plants were grown in randomised plots ( $1 \times 3\text{m}$ ), growth analysis was made by standard procedure.

## RESULTS

*Study of the rate of deposition of air borne particles and its microscopic analysis.*

The results as depicted in the Figure 1 indicate that depending on the weather condition the rate of deposition of particles is extremely variable. It varies from 49 kg/acre/week to 1.2 kg/acre/week. During the storm it increases to the maximum and after a heavy shower of rain it decreases to the minimum and then gradually rises up until the next shower. The Baruipur farm area is located outside the Calcutta yet the amount of deposition of air borne particles is quite heavy. The average deposition is 1000 kg/year/acre.

The microscopic analysis of the collection reveals that these particles are extremely complex in nature. The solid particles can be roughly classified into three groups : (1) Inorganic particles of sand, rock, different metals, inorganic salts etc. (2) Organic particles of coal, soot, rubber, various organic matter etc. (3) Biological particles of spore, pollen, mycelium fibre etc. In many cases these three components closely adhere together forming a single particle which makes them heavy enough and ultimately deposited on the ground.

Sizes of these particles vary from 1000  $\mu$  to 0.1  $\mu$ , complex particles (those composed of more than one components) are invariably multicoloured in nature (Plate 1; Fig.1) . The percentage of distribution of three individual components per microscopic field has been measured. From the outward shape and structure the pollen grains of different plant species have been identified.

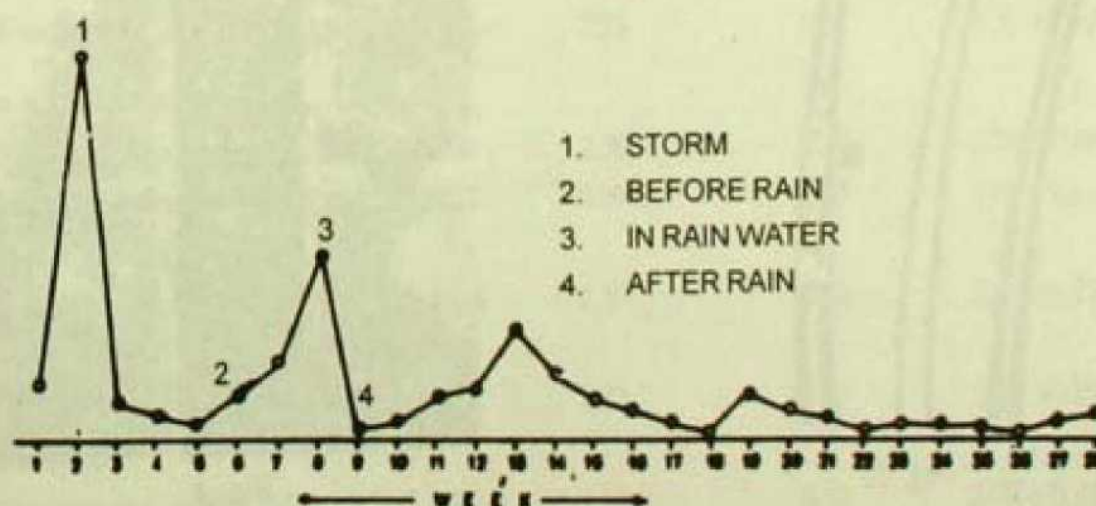


Fig. 1. The rate of deposition of air borne particles in Calcutta University Experimental Farm at Baruipur.



Fig. 1.



Fig. 2.

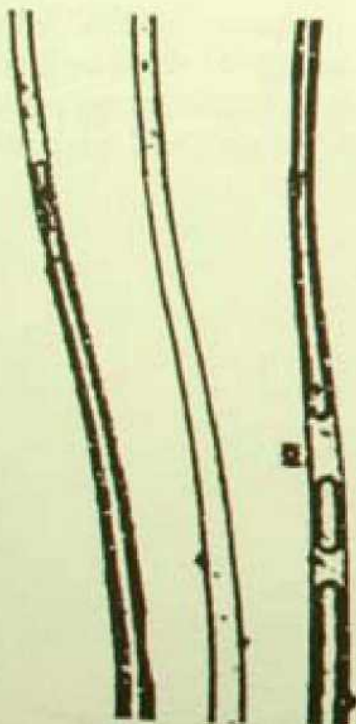


Fig. 3.

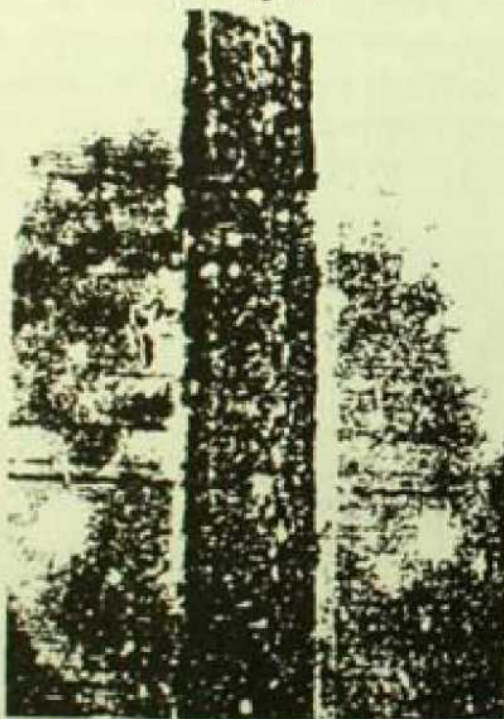


Fig. 4

Plate 1

- Fig. 1. Complex air borne particles consisting of coal soot and crystals of organic and inorganic compounds.
- Fig. 2. Photomicrograph of auricular hairs of rice plant with accumulation of dust particles on their surface.
- Fig. 3. Presence of water and gas bubbles in auricular hairs.
- Fig. 4. Formation of crystals of calcium oxalate in the auricular hair after treating subsequently with  $\text{CaCl}_2$  and oxalic acid.

**Table 1. Dust filtering efficiency of different species of avenue trees. (Samples were collected from Indian Botanical Garden, Shibpur, Howrah in the month of February)**

	Dust/sq m of upper leaf surface (g)	Dust/sq m of lower leaf surface (g)	Total dust/sq m of leaf surface (g)
<i>Trees with simple leaf</i>			
1. <i>Ficus religiosa</i> L. Peepal (Aswatha)	2.56	1.59	4.15 ± 0.07
2. <i>Ficus infectoria</i> Roxb. Pakur	2.64	1.45	4.09 ± 0.08
3. <i>Ficus benghalensis</i> L. Banyan	2.71	0.88	3.59 ± 0.06
4. <i>Teclona grandis</i> L. f. Teak	4.10	1.25	5.35 ± 0.12
5. <i>Shorea robusta</i> Gaertn. Sal	3.40	1.10	4.50 ± 0.09
6. <i>Terminalia arjuna</i> Bedd. Arjuna	3.25	1.24	4.49 ± 0.10
7. <i>Polyalthia longifolia</i> Benth & Hook. Mast	3.92	0.64	4.56 ± 0.09
8. <i>Mangifera indica</i> L. Mango	2.50	1.55	4.05 ± 0.06
9. <i>Lagerstroemia flosreginae</i> Ritz. Jarul	2.82	1.22	4.04 ± 0.03
10. <i>Bauhinia purpuria</i> L. Kachnar	2.70	1.20	3.90 ± 0.13
11. <i>Anthocephalus cadamba</i> Miq. Kadamba	2.42	1.15	3.57 ± 0.08
12. <i>Thespesm populnea</i> Soland. Tulip tree	2.82	0.71	3.53 ± 0.07
<i>Trees with compound leaf</i>			
13. <i>Saraca indica</i> L. (Asoka)	2.56	1.22	3.78 ± 0.08
14. <i>Butea frondosa</i> Roxb. Flame of the forest (Palas)	2.20	0.85	3.05 ± 0.14
15. <i>Azadirachta indica</i> A. Juss Margosa (Nim)	2.20	0.72	2.92 ± 0.13
16. <i>Cassia fistula</i> L. Indian laburnum. (Sondal)	1.82	0.42	2.24 ± 0.19
17. <i>Tamarindus indica</i> L. Tamarind	1.56	0.52	2.08 ± 0.08
18. <i>Poinciana regia</i> Bojer. (Syn. Delonix regia Raf.). Gold mohur (Gul mohor)	1.12	0.32	1.44 ± 0.07

Figures are average per ten replication.

**Table 2.** Estimation of different elements present in dust samples collected from the leaf surface of *Ficus religiosa* L. of Cossipore area, Calcutta.

Name of elements	% present
Carbon (organic)	5.19
Nitrogen	0.63
Phosphorus	0.30
Potassium	0.41
Iron	0.22
Manganese	0.04
Copper	0.02
Zinc	0.14
Calcium	3.40
Magnesium	0.41
Silicon	22.00
Traces of Cl, Pb, As, Ba, Cd, Al	

### Dust filtering capacity of different avenue trees of India

Aerosol consists of solid particles in the form of dust, fumes or smoke and of liquid molecules in the form of mist or fog. It includes any particle larger than single molecule but small enough to remain dispersed in the air for a significant length of time. This aerosol has great affinity to adhere to any solid surface when comes in contact with it. When the air current passed through a tree, some of solid suspended particles adhere to the upper or lower surface of leaves, some of them are bounced back and became air borne again or deposited on the base of the tree depending on the size and character of the particle, velocity of wind and the surface of contact. In order to study the dust filtering capacity of different avenue trees, leaves of different species growing in the same area (Botanic Garden, Calcutta) and approximately of same age were carefully collected from approximately same height from ground level in the month of February and deposition of dust per unit area of the leaf surface was measured by removing the dust particles from the upper as well as lower surface of the leaf with the help of very clean camel hair brushes and weighing them with chemical balance upto fifth place of decimal.

A list of Indian avenue trees has been prepared in order of their dust collecting capacity. Table 1 indicates the comparative dust collecting efficiency of some of the common species of trees and ornamental plants which are planted on the roadsides in India. It is revealed that evergreen trees with simple leaves, rough or hairy surface are better dust collectors than those of deciduous trees with compound leaves. In this regard *Ficus*, *Mangifera*, *Tectona*, *Polyalthia* are better dust collectors than *Cassia*, *Poinciana* or *Sesbania*. It is of interest to note that the upper surface of the leaf collects most of the dust particles; but the lower surface also collects an appreciable amount.

The dust sample which was collected from the leaf surface of avenue trees of Calcutta was subjected to chemical-analysis. Table 2 reveals the relative amount of the elements that have been detected in the dust. The presence of major and micro nutrients of plants as well as toxic chemicals like Al, Cd, As, Pb are also found. These chemicals obviously play significant role in metabolism of the tree.

### Study on the effect of deposition of particles on growth of paddy plants

Boro paddy var. Ratna was grown at the randomised plots in the Calcutta University Experimental Farm and three sets of treatment were given: (1) The dust collected from the surface of the farm soil was sprinkled on the surface of the rice leaves at the rate of 1 kg/3 sq m/week. (2) The surface of the leaves were cleaned carefully with the help of a feather duster and kept as control. (3) Another set of treatment was taken in which plants were left as untreated control.

**Table 3. Effect of dusting on the growth and yield of rice plants.**

Treatment the Plant (cm)	Height of leaves/ plant	No. of particle (cm)	Length of the particle (g)	Wt. of the grains weight (g)	Thousand
DUSTING	66.4±	14.3±	23.4±	15.1±	15.500
Mean ± S.E.	0.924	0.496	0.267	0.837	
CLEANING	67.5±	16.4±	22.2±	23.7±	18.200
Mean±S.E.	1.184	0.642	0.449	1.211	
NORMAL	68.9±	15.1±	22.1±	27.3±	18.900
Mean±S.E.*	1.341	0.612	0.337	0.898	

It reveals from the result that sprinkling of dust particles on the surface of rice leaf does retard the yield and accumulation of dry matter in the leaf, when it is compared with the control (Table 3). The same result was obtained at different ages of the plant, which indicates that the result is consistent. It is of interest to note that cleaning of rice leaves did not encourage the growth and yield of crop as compared to that of untreated control, on the other hand the height of the plant as well as total weight of the panicle had been slightly decreased by cleaning treatment. It shows that the normal rate of deposition of air borne particles on the rice plants does not adversely affect the growth and yield, but it significantly retards the growth and yield of the crop when it exceeds a certain threshold limit.

### Mode of absorption of air borne particles by cereals

Unlike dorsiventral leaves of dicotyledonous plants, monocotyledonous grasses including cereals have erect structure of leaves (isobilateral) and consequently a considerable amount of solid particle fallen on the leaves slides down and deposited at the base of the leaves (lamina). At the base of the leaves copious and numerous unicellular hairs are present which are called the auricular hairs (Plate 1; Fig. 2, 3). These are the modified structure of legule and each is connected with vascular supply. Microscopic observation reveals a dense accumulation of dust particles on the surface of these hairs (Plate I; Fig. 2). Physiological function of these auricular hairs is little

known and no significant physiological study has so far been made on this organ. During the course of present investigation it is revealed that the principal function of these unicellular hairs is to absorb water and salt solution. These unicellular hairs are extremely active in absorption of water or salt solution, which can be demonstrated under the microscope by mounting a microscopic section of the leaf base on a slide and then adding a drop of water or colouring matter like neutral red with 0.005 M  $\text{CaCl}_2$  solution to the tip of the hair. These hairs act like needles of hypodermic syringe sucking the water or colouring matter at a very rapid rate (Plate 1; Fig. 3). The gaseous exchange also takes place through these hairs which is revealed from the appearance of air bubbles in the liquid column inside the hairs (Plate 1; Fig. 3). When the hairs were treated subsequently with  $\text{CaCl}_2$  solution and oxalic acid, the crystals of calcium oxalate were formed inside the hairs (Plate 1; Fig. 4) including the uptake of both the solutions by these hairs.

## DISCUSSION

The results show that quite a large amount of air borne dust particle deposits on the crop field, the rate of which increases during winter and summer months and decreases during rainy season. Avenue trees by offering enormous leaf surface collect an appreciable amount of dust particle, amount of which varies from species to species. Trees with evergreen, simple leaves are better dust collectors than trees with deciduous, compound leaves. In Russia a parallel study (2) showed that among avenue trees lilac is the most efficient dust collector which collects 2.33 g of dust per square metre of leaf area. In the present study the best rank of dust collector includes *Tectona grandis* L.f., *Mangifera indica* L., *Ficus religiosa* L., *Shorea robusta* Gaertn., *Terminalia arjuna* Bedd., *Polyalthia longifolia* Benth and Hook., and *Lagerstromia flosreginae* Ritz. which collect 5.35 g/m<sup>2</sup> to 4.04 g/m<sup>2</sup> dust particles during the winter season.

The dust collecting capacity of trees with compound leaf or needle shaped leaf (pine trees) is rather poor. But those leaves also exert a significant filtering effect when air current passes through their dense formation. They act like sieves and separate the suspended particles of the air by offering physical obstruction with considerable mechanical advantage.

The microscopic analysis of air borne particles deposited on the leaf reveals that in most cases these particles form conglomeration, i.e., a number of particles gathered into clow or mass and in many cases they are partially or completely covered with black soots. Such soots contain a number of chemicals which are carcinogenic in nature. The chemical analysis of particles show the presence of major and micro elements as well as toxic and inert substances. The study of foliar application of chemicals (8) indicates that although in trees, a waxy cuticle is formed on the outer epidermal walls of the stems and leaves yet they too can absorb chemicals applied to their leaf surfaces. The stomata and the presence of cracks in the cuticle and the possible occurrence of plasmodesmata extending from the epidermal cells into the cuticular layer may explain the pathway for chemical transport into the leaves. Hence the role of air borne major and micro elements in the metabolism of the plants and the adverse effects of toxic chemicals on plants and subsequently on animals including human beings cannot but be ruled out.

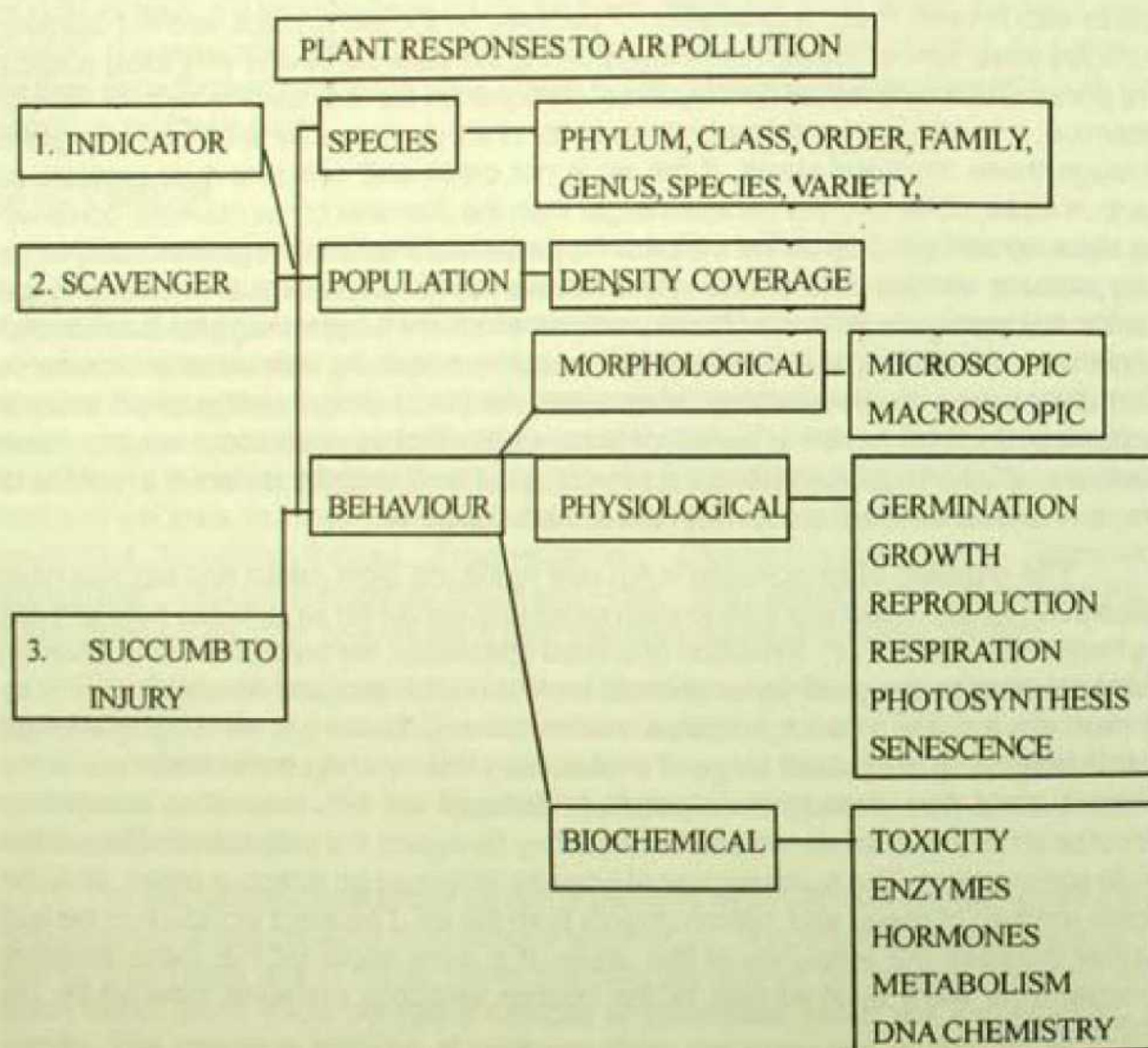
Most of the Gramineous species including cereals possess copious and numerous auricular hairs at the junction between the leaf blade and leaf sheath. The result shows that auricular hairs occupying a strategic position at the base of the lamina can absorb various chemical compounds from air borne particles that are deposited on their surface. During the metabolic process of the plant the carbonic acid is continuously formed from water vapour and  $\text{CO}_2$  coming out through these hairs and with the help of the water and carbonic acid some of the chemicals accumulated on the surface of the hairs are dissolved and ultimately absorbed by the plant.

Microscopic study of the leaf surface after sprinkling of dust particles on the rice plants also reveals that the deposition of particles on the leaf surface was not uniform, particles were concentrated more heavily on the stomatal pores and very thinly outside the pores. Such differential distribution of particles on the leaf surface requires special attention. It is well known that a large quantity of air continuously enters into the plant through these stomatal pores, if the air is not clean and contains dust particles of various sizes, obviously the particles larger than the diameter of the stomatal pores will be stuck-up and piled-up on the orifice of the pores and interfere the gaseous exchange and produce various deleterious effects on the normal physiological functions of the plants. It is also quite likely that those particles which are large enough for the stomatal pores may ultimately get access inside the leaf by dissolving with water and carbonic acid discharged by the stomata themselves. As the chemical nature of particles is extremely diverse, hence a series of synergistic reaction may occur among these particles, which in turn, can affect the physiological functions of the plant in a number of ways. A further detailed study may reveal the full picture.

The problem of air pollution is not new to life. Both plants and animals have been evolved with adequate built-in-mechanisms to encounter air pollution even as early as from mesozoic era (4). Evolution of ciliated epithelium, the protective lining covering the respiratory passage of higher animals and the nostril hairs and the structure of nose of man are the few of such adaptive mechanisms. Grasses are also highly evolved plants that came at the later stage of evolutionary history when the atmosphere of the ancient world had already been polluted. Grasses not only are more resistant to adverse environmental conditions but also they do exploit the polluted condition of the air to some extent. The auricular hair of grasses, acting as an adaptive organ, absorbs some amount of major and micronutrients from the air. The erect structure of the leaf further increases the efficiency of this organ. It is quite apparent that these adaptive mechanisms were evolved due to the intense selection pressure induced by the pollutant itself.

A parallel example of utilization of polluted condition of the air by the plant can be cited from orchids. Orchids are also highly evolved plants and they appeared at the more recent period of evolutionary history. Orchidaceae family with its 25,000 species comprises the largest family among the flowering plants. Most of the members being epiphytes live on exploiting the polluted condition of the air. They collect all their major and micronutrients from the air. Epiphytic habit is not the monopoly of orchids alone, some members of Bromeliaceae and Aroideae are also epiphytes. If the air of ancient world were perfectly clean the evolution of these epiphytes would not have been possible.

These grasses, epiphytes and tall trees by offering various self-cleansing mechanisms have been maintaining the purity of air through ages and thereby maintaining the balance of nature. But the self-cleansing mechanism has always some definite threshold limit. At some degree of accumulation of toxic chemicals in the plant the self-cleansing powers are exhausted. It is, therefore, left to us to find out these threshold values with different species of plants with different types of pollutant and their synergistic effects on various physiological functions. The researches on this line would help us to formulate the strategy of survival of life on an infinite time scale.



### Formula for measuring Lichen Growth

The increase in radial growth was expressed as increment of radius from a lichen of 100 mm thallus circumference taken as a unit in the format.

$$RADIAL\ GROWTH = 15.9155 \left( \sqrt{\frac{A_2}{A_1}} - 1 \right) mm$$

Where 15.015 is the initial radius of a lichen thallus of 100 mm circumference  
 $A_1$  and  $A_2$  refers to area of lichen thallus measured at initial and final readings.

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# Oxidative Stress and Brain Aging

DR. SASANKA CHAKRABARTI

Aging is an intrinsic process which affects cells and tissues to produce a state in which the fitness of an organism is grossly impaired. Several theories such as somatic mutation and DNA damage theory, protein-error theory and antagonistic pleiotropy theory have attempted to explain the genetic mechanisms involved in aging. However, there are many epigenetic factors which profoundly affect the phenomenon of aging and the oxidative stress is considered to be one such critical epigenetic mechanism. The importance of oxidative stress in the aging process is indicated by several recent discoveries. Thus, an over expression of anti-oxidant enzymes increases the life of transgenic *Drosophila melanogaster* and the susceptibility to oxidative stress and the levels of anti-oxidant enzymes correlate nicely with the organism life span. Moreover, caloric restriction reduces the oxidative stress and extends the life-expectancy of the organisms.

In the context of brain aging, the importance of oxidative stress cannot be over emphasised because of special vulnerability of brain to damaging effects of reactive oxygen species (ROS). Thus, of all the organs, the brain has the maximum oxygen consumption per gram of tissue and it is enriched in oxidizable substrates (poly-unsaturated fatty acids, catecholamines etc.) and transition-metals. The antioxidant enzyme catalase is deficient in brain while SOD and glutathione peroxidase are present in moderate amounts. All these factors tend to make the brain tissue highly vulnerable to oxidative stress. In the aging brain, this is further compounded by an enhanced production of ROS, a decline in anti-oxidant defence and increased peroxidizability of brain membranes.

The reactive oxygen species consist of several members such as superoxide radical ( $O_2^{\cdot-}$ ),  $H_2O_2$ , hydroxyl radical ( $\cdot OH$ ) and singlet oxygen which are formed from endogeneous metabolism. The major source of superoxide radicals is mitochondrial ETC. Hydrogen peroxide is generated from spontaneous or SOD-catalyzed dismutation of  $O_2^{\cdot-}$  radicals or from the action of MAO or L-amino-acid oxidase. Superoxide radicals and  $H_2O_2$  interact in presence of transition metals like  $Fe^{2+}$  or  $Cu^+$  to produce highly reactive  $\cdot OH$  radicals. The sources and interactions of different members of ROS are depicted in Fig. 1.

ROS can avidly attack various bio-molecules including phospholipids, proteins and DNA. The reaction involving unsaturated fatty acid moieties of phospholipids is termed lipid peroxidation and results in the formation of several toxic aldehydes like

malondialdehyde and 4-hydroxynonenal. The detailed mechanism is depicted in Fig 2. The protein damage consists in amino acid modifications, protein fragmentation or cross-linking, formation of protein carbonyls and hydroperoxides and various other changes. The damage to DNA gives rise to modified bases (8-hydroxy-deoxyguanosine, FAPya etc.), formation of DNA-adducts and fragmentation of DNA strands. The major oxidative lesions in DNA are listed in Table 1. At the cellular level, such damage to various molecular targets is reflected as changes in membrane properties, inactivation of enzymes and receptors, loss of  $\text{Ca}^{2+}$  homeostasis, mitochondrial dysfunction and activation of apoptosis.

The aged brain even in the absence of any associated neurological disease presents characteristic morphological and histological changes such as gyral atrophy, neuronal loss and reduced synaptic connectivity with glial reactivity. This is associated with an accumulation of oxidation products of lipids, protein and DNA in the aged brain. An increased membrane peroxidation, accumulation of lipofuscin pigments and fluorescent lipid peroxidation products, elevated levels of protein carbonyls and nitrotyrosine and a loss of protein thiols have all been reported in brain during aging. The oxidative modifications of proteins and lipids are accompanied by inactivation of several enzymes glutamine synthetase, multicatalytic proteasome, ( $\text{Na}^+$ ,  $\text{K}^+$  - ATPase etc.), transport proteins (glutamate transporter) and receptors along with alterations of membrane potential and other membrane properties. Various kinds of oxidative DNA lesions have been shown to be elevated in aged brain. This includes DNA-protein cross-links, MDA-DNA adducts and various types of 1-Compounds, but it is debated whether the level of 8-hydroxydeoxyguanosine, the most-popular marker of oxidative DNA damage is increased or not in the aged brain. There are scattered reports of mitochondrial dysfunction, loss of calcium homeostasis and activation of apoptotic mechanisms in aged brain which have been attributed to oxidative stress.

Although the evidence in favour of oxidative damage in brain aging is overwhelming, it has not so far been possible to identify characteristic oxidative lesions in specific target proteins like ion-channels, receptors or transport proteins in aged brain. Further, it is not clear how on accumulation of oxidized products of lipids protein or DNA can lead to neuronal dysfunction and functional impairment of brain during aging.

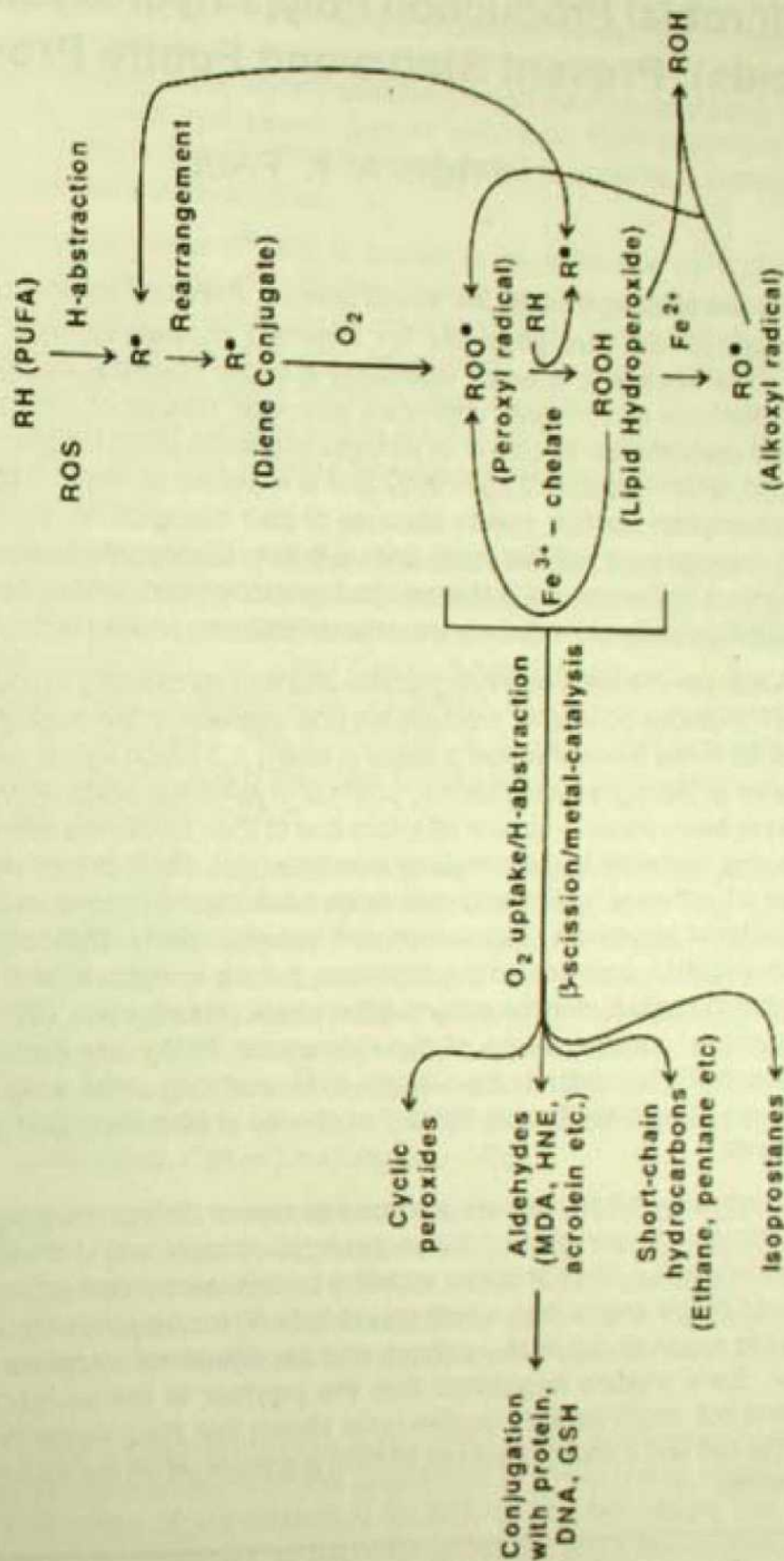
**Table 1**

### **Oxidative DNA Lesions**

- Hydroxylated derivatives of G or A, 8-hydroxydeoxyguanosine ( 8-oxodG)
- Modified A or G with ring opening e.g. FAPY<sub>G</sub>, (2, 6 diamino-4 hydroxy, 5-formamidopyrimidine)
- Modified pyrimidine bases e.g. thymine glycol, cytosine glycol
- Modified deoxyribose e.g. carbonyl derivatives
- Type II 1-compounds
- Exocyclic DNA adducts
- DNA fragmentation and single-strand breaks
- DNA-protein cross-links



Fig. 2  
 Outline of Lipid peroxidation



## Microbial Production Poly(3-hydroxyalkanoic acids): Present Status and Future Prospects

PROF. A K PAUL

We are passing through the age of plastics. Plastics have become an integral part of our everyday life for inherent properties like, light weight, imperviousness to water, insolubility in water, chemical stability, non-toxicity, inertness to corrosive chemicals and wide degree of structural versatility. Per capita consumption in India of all varieties of plastics taken together was about 2.4 kg in 2000, which raised to 3 kg in 2002 and is expected to rise to 5 kg by 2010. The post consumption plastics, mainly because of their biological resistance and lack of efficient management policies finally end up in non- biodegradable waste stream and cause serious environmental problems. Biodegradable plastics have been identified as one of the alternatives to reduce environmental pollution caused by thermoplastics.

A number of inherently biodegradable plastics representing a range of properties suitable for various consumer products are now available in the market and the global demand for these biodegradable plastics is nearly 1.3 billion kg per year. Among the candidates of biodegradable plastics, polyhydroxyalkanoic acids (PHAs) of bacterial origin have been drawing special attention due to their properties similar to synthetic plastics and complete biodegradability in nature (Doi, 1990; Brandl *et al.*, 1990). As many as 91 different hydroxyalkanoic acids have been detected as constituents of these bacterial polyesters (Steinbuchel and Valentin, 1995). The composition of the accumulated PHA depends on the organism, culture conditions, and carbon source provided to the cells during the accumulation phase (Steinbuchel, 1991). According to the number of carbon atoms of the monomers, PHAs are classified as short chain-length PHA, medium-chain-length PHA and long-chain length PHA. A few bacteria are able to synthesize PHAs that consist of both short and medium-chain-length acids.

Polyhydroxyalkanoates are produced as natural storage compounds in bacteria when their growth is restricted by an essential nutrient and if a carbon source is available in excess. They occur as inclusion bodies surrounded by an interface layer composed of lipid and protein, which serves not only to compartmentalize the polymer but also to organize the PHA synthase and depolymerase enzymes in a functional manner. Early studies suggested that the polymer in the inclusion bodies was crystalline but more detailed studies have shown that the polymer is in a fluid state inside the cell and it only crystallizes to form a granule when the inclusion is released from the cell.

Though polyhydroxyalkanoates are water-insoluble, hydrophobic and partially crystalline polymers, they can be degraded in the environment by a variety of microorganisms by the production of extracellular depolymerases (Jendrossek *et al.*, 1996). Polyhydroxyalkanoate- degrading bacteria have been isolated and characterized from terrestrial and aquatic ecosystems and the PHA-degrading bacteria have been distinguished into eleven groups based on their substrate (PHA) specificities. The ability to degrade PHA is not restricted to bacteria and many PHA-degrading fungi have also been identified.

Polyhydroxyalkanoates (PHAs) is known to be produced by hundreds of different bacterial species. Only a few bacteria having the ability of accumulating PHA to a significant level have been employed for mass production. These include members of *Alcaligenes* (*Ralstonia*), *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Rhizobium*, some methylotrophs, phototrophic bacteria and recombinant *Escherichia coli*. Strategies for growing these bacteria and to obtain high concentration of PHA have been developed and conditions required for efficient PHA accumulation have been identified for some of them.

Metabolic pathways leading to the synthesis of PHAs have been studied in details for a few bacteria with increasing emphasis on the enzymology and control of these pathways. The key enzyme of PHA biosynthesis is PHA synthase. Majority of bacteria synthesize poly(3HB) from acetyl-CoA via 3-hydroxybutyryl-CoA, employing a three-step pathway with  $\beta$ -ketothiolase, an NADPH-dependent acetoacetyl-CoA reductase and Pha synthase. The Acetyl-CoA is a central intermediate in the metabolism of any organism and is therefore formed not only from carbohydrate and fatty acids but from any carbon source.

Carbon sources that are degraded via acetoacetyl-CoA or 3-hydroxybutyryl-CoA will shortcut the pathways for PHAs. The synthesis of PHAs other than poly(3HB) occurs only from precursor substrates in most microorganisms. These are structurally related to the constituents that are to be incorporated into PHAs and fed as the sole carbon source or as a cosubstrate. The synthesis of these PHAs depends on the level of metabolism needed to convert the precursor substrates into their corresponding hydroxyacyl-CoA thioesters. Synthesis of copolymer, poly(3HB-co-3HV) occurs in most poly(3HB)-accumulating bacteria only from substrates that can be converted into propionyl-CoA, 3-ketovaleryl-CoA or 3-hydroxyvaleryl-CoA.

The copolymers of PHA have improved mechanical properties making them suitable replacement of petrochemically produced bulk plastics. But the major problem that prevents the commercial application of PHAs is their high price. Much effort has been devoted to reduce the price of PHAs by the development of better bacterial strains, use of cheaper substrates, more efficient fermentation and more economical recovery processes.

The cloning and expression of PHA biosynthetic genes in a foreign host have achieved significant advancement in the production of PHA. Using recombinant bacteria, PHA production of approximately 80-90% of dry cell weight has been recorded. Similarly, the availability of bacterial genes for PHA biosynthesis and knowledge of their structures, as well as the structure of PHA inclusions and availability

of sophisticated methods of plant biotechnology, have enabled the production of PHAs in transgenic plants. The initial demonstration of transgenic *Arabidopsis thaliana* expressing PhaB and PhaC proteins (from *R. eutrophus*) in their cytoplasm resulted in transgenic plants with low levels of poly(3HB); the plants also exhibited retarded growth. However, when all three *R. eutrophus* poly(3HB) -biosynthesis genes (phaA, phaB and phaC) were expressed in the chloroplast of *A. thaliana*, the transgenic plants exhibited normal growth and had a poly(3HB) content of approximately 14% of the dry weight, with leaves showing slight chlorosis after prolonged growth. Recently, the *R. eutropha* genes have also been successfully expressed in *Gossypium hirsutum* and *Zea mays*; experiments for expression in other relevant agricultural crops are under way in several laboratories. PHA biosynthesis by *in vitro*, biosynthetic routes and by purely chemo synthetic routes also is being actively pursued. However, until recently PHA production for commercialization mostly rely on efficient bacterial fermentation.

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# Metabolic Regulation of Photosynthesis

PROF. S MUKHERJI

**T**he Calvin cycle is divided into three sections, (i) carboxylation, (ii) reduction of 3-PGA to triose phosphate, (iii) the regeneration of  $\text{CO}_2$  acceptor molecule RuBP, and (iv) the conversion of triose phosphate to carbohydrate end product. Starch is synthesized in the plastid from fructose 6-phosphate, which is withdrawn from the regeneration phase of Calvin cycle. Sucrose is synthesized in the cytosol from triose phosphates which exit Calvin cycle before plastid fructose 1,6 - biphosphatase (pFBPase). There is a strict coupling of triose phosphate export and phosphate uptake because it is facilitated by triose phosphate translocator (TPT) and catalyzes a strict counter exchange.

## REGULATORY PROPERTIES OF CALVIN CYCLE ENZYMES

**A. Light Drives Photosynthesis by Activating Several Calvin Cycle Enzymes via Thioredoxin and Changes of Stromal pH and Magnesium :** Illumination isolated chloroplasts of leaves leads to alkalization and increased free  $\text{Mg}^{2+}$  in stroma. Several enzymes of the Calvin cycle are strongly activated by increasing pH and  $\text{Mg}^{2+}$  including pFBPase, SBPase and RuBisCO. The pH and magnesium sensitivity of pFBPase is due to an increase in the proportion of the total FBP pool present as  $\text{FBP}^{4-} - \text{Mg}^{2+}$  which is the real substrate. An analogous explanation is possible for SBPase, pFBPase, PRK and NADP-GAPDH in Calvin Cycle. An enzyme termed ferredoxin -thioredoxin oxidoreductase transfers reducing equivalents from ferredoxin to a small soluble protein called thioredoxin. Thioredoxin then modifies cysteine groups on the target protein. Further enzymes outside the Calvin cycle including NADP malate dehydrogenase and CF<sub>1</sub> ATP synthase in thylakoids are also activated by thioredoxin. The precise effect of thioredoxin activation of these enzymes is an increased substrate affinity.

**B. Substrates Modulate Thioredoxin Activation to Allow Feedforward and Feedback Coordination of Calvin Cycle Enzymes :** The increased activation occurs because substrate modifies the mid-redox point potential of the cysteine groups on the enzymes, thus shifting the extent to which they can be reduced at a given reduction state in the chloroplast. For FBPase activation can be promoted by adding FBP, for PRK, by ATP.

This modulation of thioredoxin activation by substrate levels can be viewed as a feedforward regulation, which allows a sharp increase in enzyme activity when substrate concentration rises, and restricts activity when substrate concentration falls.

**C. Product Inhibition and Feedback Inhibition Provide Additional Coordination of Calvin Cycle Fluxes :** Each of these enzymes is also inhibited by its products, or by intermediates occurring later in Calvin cycle. The pFBase is inhibited by F6P, SBPase by S7P, PRK by both products, i.e. ADP and Ru5P. These feedback inhibition loops complement the feedforward loops described before.

**D. Coordination of ATP and NADPH Production is Evident :** Photosynthesis requires formation of NADPH and ATP in required stoichiometry. Besides regulation of photosynthetic electron transport, there is an additional mechanism in carbon metabolism which acts to balance ATP and NADPH formation.

A shortage of ATP will lead to an accumulation of NADPH. When NADPH accumulates and NADP declines, NADP- MDH is activated. This allows NADPH oxidation in the stroma, which in turn will allow more linear electron transport and ATP synthesis in the thylakoids. Malate is exported to the cytosol and is oxidized outside the chloroplast by NAD - MDH.

The modulated 'malate valve' provided by NADP-MDH is important for extrachloroplastic metabolism, because it is a source of NADH, and potentially ATP. NADH is required to drive nitrate reduction in cytosol. Some of the malate may enter peroxisomes, instead of being oxidized in cytosol. Oxidation via NAD-MDH in peroxisome provides NADH for hydroxypyruvate reductase in photorespiratory pathway. This will allow some NADH formed during glycine decarboxylation to be retained in mitochondria rather than shuttling it to peroxisome to support hydroxypyruvate reduction. As a result, NADH can be oxidized in mitochondria to provide additional ATP, which can be used for sucrose synthesis, for transport processes, and could even be used together with NADH to allow some of the PGA to be reduced in cytosol, instead of in chloroplast.

**E. The Unique Importance of Rubisco :** Rubisco catalyzes the key reaction in which RuBP acts as an acceptor for  $\text{CO}_2$  forming 2 moles of PGA. The competing reaction with  $\text{O}_2$  leads to the formation of 2-phosphoglycolate which is recycled via photorespiratory pathway. Due to the relatively high  $K_m$  for  $\text{CO}_2$  and the competing reaction with  $\text{O}_2$ , Rubisco is  $\text{CO}_2$ - limited in ambient conditions. The relative efficiency of the enzyme is partly counterbalanced by the very high amount (up to 30%) protein in leaf.

Relatively poor affinity for  $\text{CO}_2$  also entails a high stomatal conductance during rapid photosynthesis, resulting in increased transpiration. Rubisco regulation will be of crucial importance in determining how effectively this unavoidably large investment in a single protein can be exploited.

**Regulation via RuBP availability :** Rubisco has a high affinity for RuBP. Leaves contain a large pool of RuBP and active sites of Rubisco are likely to be saturated with RuBP.

**Competition of other phosphorylated intermediates with RuBP :** A large number of Calvin cycle intermediates like FBP, NADPH, 6-phosphogluconate, P and PGA bind to the active site of Rubisco. However, only PGA is of physiological significance in photosynthesis.

**Carbamylation (Activation) State of Rubisco** : Rubisco is unique in that the catalytic site of the active enzyme form contains a carbamylated lysine to which a  $Mg^{2+}$  is complexed (termed ECM form). The enzyme can be carbamylated or decarbamylated *in vivo*, leading to change in 'activation state'. Activation state of Rubisco increases as irradiation increases. A protein called *Rubisco Activase* is needed to enable these changes. Rubisco activation is regulated to achieve a balance between RuBP formation and consumption, such that the active Rubisco sites (ECM) are near saturated by RuBP.

**Carboxyarabitol-1-Phosphate** : Leaves of some species contain an unusual sugar phosphate, carboxyarabitol 1 -phosphate (CAP). This is a closely related structure to 2-carboxy-3-keto-arabitol 1,5-bisphosphate, which is the transition state analog of carboxylation reaction. CAP binds with high affinity to the ECM form of Rubisco and depresses  $V_{max}$ . Leaves contain considerable amount of free sugar Carboxyarabitol (CA) in light and dark. CA acts as precursor of CAP and CAP is converted back to CA after illumination. Rubisco activase releases CAP from ECM-CAP complex.

**F. Coordination of Calvin Cycle and Photorespiration** : Rate of 2-phosphoglycolate formation is about 1/10 of the rate of PGA formation during photosynthesis at 20°C and ambient  $CO_2$ . It will be higher if temperature rises or intercellular  $CO_2$  falls due to stomatal closure. Two mechanisms for coordination have been proposed. Firstly, Rubisco activation can be inhibited by glyoxylate. Secondly, glyccrate is a powerful noncompetitive inhibitor of pFBPase and SBPase.

Feedforward regulation coordinates sucrose synthesis and  $CO_2$  fixation. Rising rates of photosynthesis lead to (i) stimulation of cytosolic FBPase caused by decrease in concentration of inhibitory regulatory metabolite fructose 2, 6-bisphosphate and (ii) activation of sucrose phosphate synthase (SPS). In both cases, the result is increased substrate affinity.

**A threshold triose phosphate concentration sucrose synthesis with Calvin cycle turnover** : Sucrose synthesis is inhibited below a critical threshold concentration of triose phosphate which will permit Calvin cycle turnover. Triose phosphate concentration must remain high enough to (i) support aldolase and transketolase reactions of Calvin cycle and (ii) generate adequate stromal FBP to allow activation of stromal FBPase. A correct relationship between the kinetic properties of plastid and cytosolic FBPase will be important in allowing stable operation of Calvin cycle.

## PHOTOINHIBITION OF PHOTOSYNTHESIS

Photoinhibition is a physiological stress condition induced in plants as a consequence of an imbalance between the number of photons captured and their utilization by photosynthesis. Photosynthetic activity is depressed in excess light affecting both plant growth and productivity. The most vulnerable part of photosynthetic apparatus is Photosystem II (PS II) which powers water oxidation and oxygen evolution. PS II acts as a water-plastoquinone reductase which has a 4-electron gate on its oxidizing side and a 2-electron gate on its reducing side. Although PS II is a multipetide complex with more than 20 subunits, these redox reactions are restricted

to only 2 polypeptides, D1 and D2 proteins and these proteins form the reaction centre of PS II.

During light-induced damage of PS II, a key component of its reaction centre. D1 protein turns over far more rapidly than any other protein in the photosynthetic membrane. This turnover is part of a repair system that functions to replace damaged reaction centers with newly synthesized D1 protein and thus restore normal PS II activity. If rate of repair of PS II does not keep pace with its rate of damage, then photoinhibition is observed as a decrease in photosynthetic activity. When the repair system copes with the rate of damage, then no loss of photosynthetic capacity is observed. The amino acid tyrosine that acts as an intermediate between manganese cluster and P680 is lying on D1 protein.

*Photochemical processes giving rise to damage* — Initial damage to PS II involves photochemical processes that inactivate its function and trigger D1 and D2 proteins for enzymatic degradation. Two distinct pathways by which photo damage may occur are one induced from acceptor side of PS II and another from donor side of PS II.

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# Plant Biodiversity : Principles, Resources and their Conservation

PROF. N. D. PARIA

**T**he natural world is a far different place now than it was 10,000 years ago, or even 100 years ago. Every natural ecosystem on the planet has been altered by humanity, some to the point of collapse. Vast numbers of species have gone prematurely extinct, natural hydrologic and chemical cycles have been disrupted, billions of tons of topsoil have been lost, genetic diversity has eroded, and the very climate of the planet may have been disrupted. What are the causes of such vast environmental changes ? Very simply, the cumulative impacts of 5.5 billion people, a number growing by 95 million each year (260,000 per day), have stressed the ecological support systems of the planet past their powers of resilience. As a consequence, biological diversity (biodiversity), the grand result of evolutionary processes and events tracing back several billion years, is itself at stake and rapidly declining. One of the many species suffering the consequences of ecological destruction is *Homo sapiens*, the perpetrator of it all.

The field of conservation biology is a response by the scientific communities to this biodiversity crisis. It is a new, synthetic field that applies the principles of ecology, biogeography, population genetics, economics, sociology, anthropology, philosophy, and other theoretically based disciplines to the maintenance of biological diversity throughout the world.

The term "biodiversity" signifies the integration of ecology and genetics in the context of conservation. The term was introduced by W.G. Rosen (quoted by E.O. Wilson, 1988) in the mid 1980's. It represents diversity at all levels of biological organisation - the community, the species, the organism, and the gene. It forms the link between the evolutionary past, through the current era of attrition and depletion, to future survival, adaptation and continuing evolution or decline and extinction.

Diversity is indeed "the essence of life" (Frankel, 1970). It is essential for survival in time and in place, and for adaptation to specific environments, in the global contexts. It has a leading role in competition, symbiosis and parasitism, the impact of climate, the absorption of nutrients and the effect of deficiencies.

Biodiversity refers to the variety and variability among living organisms, the genetic diversity they contain, the assemblages they form, and the ecological complexes in which they occur. This is auto-sustainable and self-regulating, if there are no natural and/or man-made perturbations. Biodiversity has been defined in various ways. Jutro (1995) recorded 14 different definitions of biodiversity of those most often used. Two of them - largely quoted are of a more official nature, since they have been

approved by most countries in the context of worldwide negotiations, agreements and strategies. The more extended one is that of the United Nations included in the Convention on Biological Diversity (UNEP, 1992). According to it, the biodiversity means : "The variability among living organisms from all sources including inter-alia, terrestrial, marine and other aquatic ecosystems, and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems". The shortest definition of all is that of the Global Biodiversity Strategy (WRI, IUCN and UNEP, 1992) which regards biodiversity as "The totality of genes, species and ecosystems in a region". Diversity within species is the **genetic diversity**; between species is the **species or taxonomic or organismal diversity**; and of ecosystems is the **ecological or habitat diversity**. **Genetic diversity**, is needed by any species for maintaining reproductive vitality, resistance to disease, and the ability to adapt to changing conditions. **Species diversity** represents the range of evolutionary and ecological adaptations of species to particular environments. **Ecological diversity or community level diversity** represents the collective response of species to different environmental conditions.

However, diversity may be of **alpha diversity**, **beta diversity** and **gamma diversity**. The number of species in a single community is usually described as **alpha diversity** which comes closest to the popular concept of species richness and can be used to compare the number of species in different ecosystems types. The **beta diversity** refers to the degree to which species composition changes along an environmental gradient. **Beta diversity** is high, for example, if the species composition of most communities changes at successively higher elevations on a mountain slope, but is low, if the same species occupy the whole mountain side. **Gamma diversity** applies to larger geographical scales; it is defined as "the rate at which additional species are encountered as geographical replacements within a habitat type in different localities". Thus, gamma diversity is a species turnover rate with distance between sites of similar habitat, or with expanding geographic areas. The plant communities of the Amazon, for example, show high levels of diversity at the alpha, beta and gamma scales (Gentry, 1966).

## THE EARTH SUMMIT

For 12 days in June 1992, world attention was focused on a major conference in Rio de Janeiro, Brazil, known officially as the United Nations Conference on Environment and Development (UNCED) and also as the Earth Summit, Eco '92, and the Rio Summit. Representatives from 178 countries including over 100 heads of state, plus leaders of the United Nations and major nongovernment and conservation organizations, met to discuss ways of combining increased protection of the environment with more effective economic development in less wealthy countries (United Nations, 1993a,b). The conference was successful in heightening awareness of the seriousness of the environmental crisis and placing the issue at the center of world attention (Haas et al., 1992; Alyanak, 1992). A noteworthy feature of the conference was the clear linkage established between the protection of the environment and Third World poverty. Whereas the wealthy countries of the world have the resources to provide for their citizens and protect the environment, most poor countries see the immediate use of their natural resources as the key to raising the standard of living for

their impoverished populations. At the Earth Summit, the wealthy countries were collectively made to understand the global environment and tropical biodiversity. In this Earth Summit, five major documents were signed of which the convention on Biodiversity is one of them.

**Convention on Biodiversity** : The Convention on Biodiversity has three objectives : protecting biological diversity, using it sustainably, and sharing the benefits of new products made with wild and domestic species. While the first two objectives are straightforward, the last point recognizes the developing countries should receive fair compensation for the use made of species collected within their borders. In the past industrialized countries have developed new crops, medicines, and other biotechnology products based on tropical species without returning any of the resulting technology, new products or profits to the countries in which the wild species were originally found. The treaty signed by 153 nations, affirms that countries have certain rights over species occurring within their borders. The United States at first refused to sign because of what were perceived to be potential restrictions on its enormous biotechnology industry. The United States finally signed the convention in early 1993, in the aftermath of the Clinton/ Gore victory in the 1992 election. Funding for this convention has been set initially at \$ 200 million, administered by the Global Environment Facility.

### **BIODIVERSITY IN INDIA AND AT GLOBAL LEVEL**

There are varying and often conflicting estimates floating around nationally and internationally regarding India's biodiversity. Thanks to the help of Botanical and Zoological Surveys, reliable information has been collated. The total number of living species identified in India so far is 126,188 (Table 1). With the publication of this information, speculation about the extent and nature of species richness in India should be set to rest until such time as a formal census is undertaken.

According to the World Conservation Monitoring Centre (WCMC, 1992), the total number of species described at the global level so far is 1,604,000. However, WCMC has estimated that at the global level there are likely to be 17,960,000 species, i.e. about 14 times more than the presently known species. The increase is likely to be primarily from the tropics and subtropics. However, a more realistic working figure of species at the global level is around 12,250,000 (WCMC, 1992).

Of the 126,188 species described from India (Table I), Monera (Bacteria) comprise 850 species (0.67%), Protista (Protozoa only ; minus their multicellular descendants) 2577 species (2.04%), Fungi 25,000 species (18.23%), Animalia 74,875 species (59.27%) and Plantae 24,886 species (19.79%). Nearly 72% of India's biowealth is constituted by fungi (18.25%), insects (40%) and angiosperms (13.50%). This tallies generally with the overall trend seen in the tropics and subtropics. Furthermore, although India has only 2.4% of the land area of the world as a whole, according to the present estimates, India's contribution to the global biodiversity is around 8% species (Khoshoo, 1995).

**Table 1. Number of biota in India**

Taxon	No. of species	Percentage
Bacteria	850	0.67
Algae	2500	2.00
Fungi	25000	18.23
Lichens	1600	1.30
Bryophyta	2700	2.14
Pteridophyta	1022	0.80
Gymnosperms	64	0.05
Angiosperms	17000	13.50
Protozoa	2577	2.04
Mollusca	5042	4.00
Crustacea	2970	2.35
Insecta	50717	40.00
Other invertebrates	11252	9.00
including Hemichordata		
Protochordata	116	0.10
Pisces	2546	2.02
Amphibia	204	0.16
Reptilia	428	0.34
Aves	1228	1.00
Mammalia	572	0.30
Total :	126188	100.00

Source Khoshoo, 1995.

**Global Biodiversity of Plant Kingdom**

Plant Kingdom  
(3.50.000 species)

Flowering Plants	2,50,000	species
Conifers and allied	700	"
Pteridophytes	12,000	"
Bryophytes	25,000	"
Thallophytes :		
Algae	50,000	"
Fungi	51,000	"

(Source : Adjanohoun, 1996)

## Diversity of Cultivated Plants

A group of Soviet research workers headed by N.I. Vavilov have revealed several patterns in the geographic distribution of the Earth's plant resources and identified the directions in which search for new plants, species, and varieties should be conducted. Analysis of a wealth of data collected in numerous trips undertaken in more than 60 countries of Asia, Africa, southern Europe, North and South America and also through the length and breadth of the USSR has enabled N.I. Vavilov to elaborate a comprehensive theory of world centres of origin and diversity of the major cultivated plants.

Vavilov has shown that plant species are unevenly distributed over our planet in the modern geologic epoch. Some parts of the world are characterized by an astounding diversity of species. These include south-eastern China, Indo-China, India, the Malay Archipelago, South-East Asia, tropical Africa, Ethiopia, Central and South America, the Mediterranean basin, the Near East, and some others. Northern countries and regions, such as Siberia, all of central and northern Europe, and North America, on the other hand are poor in species composition. Certain areas of the world provide striking examples of how different concentrations of plant species can be. Such small Central American republics as Costa Rica and Salvador, each occupying an area a hundred times smaller than that of the United States, have as many species as all of North America that is, the United States and Canada combined.

In most cases, a particular genus or species is associated with a single centre, but some crops are associated with two or more centres of diversity. Vavilov recognized **primary locales**, or centres, of origin, where the plant in question takes the most diverse forms and was domesticated for the first time, and **secondary centres** arising as a result of migration of individual forms from the primary one. He identified **eight** independent centres of origin of the major cultivated plants worldwide or, in other words, eight regions of domestication of various plants. These centres include Chinese centre of origin (136), Indian (Hindustan) centre of origin (117), Indo-Malay centre (42), Near Eastern centre (38), Mediterranean centre (84), Abyssinian centre (38), South American and Central American centre, South American (Peruvian - Ecuadorian-Bolivian) centre (45). In addition to the main South American centre of origin, Vavilov also recognized two sub centres: The Chiloe centre (4) and Brazilian-Paraguayan centre (13).

## SOME REASONS FOR LOSS OF BIODIVERSITY

**Global Climate Change & Biodiversity** : Such changes are expected to affect the extent and nature of forest cover and thereby of biodiversity also, through changes in population size and distribution. The reasons for this change would stem from changes in pattern of precipitation, evaporation, wind, frequency of storms, fire, sea level rise and loss of coastal wetlands and coral sites.

**Pollution and Biodiversity** : Banning the general effect of acid rain and other pollutants on forests and lake systems, the effects on biodiversity of pollution of air, water and land by toxic chemicals (including pesticides, acid due to  $\text{SO}_2$ ,  $\text{NO}_2$  etc. and ozone depletion have not been studied in detail in developing countries, including India. Therefore, attention needs to be paid to this aspect.

**Increased Use of Toxic Chemicals :** A new and powerful human threat to species diversity is the release of toxic chemicals and pesticides and herbicides into the atmosphere, soil, lakes and rivers. With increase in intensive cropping and the adoption of new varieties of commercial crops, such as vegetables and fruits, the nutrient exhaustion from soils has increased, necessitating application of high doses of chemical fertilizers to obtain remunerative yields. In the long run acidification of soils caused by continuous over-application of acidifying fertilizers also reduces productivity and adversely affects the beneficial organisms present in the soil.

**Population Growth and Extensive Farming :** Man ploughs the grassland, eliminating a hundred species of native herbs and grasses, which he replaces with pure stands wheat, maize, or barley. Although this increases efficiency, productivity and yield, but it also increases ecologic vulnerability and instability. The landscape diversity is reduced.

**Increased Stress on Forests :** Tropical forests are richer in species than any other terrestrial habitat. Tropical forests are important not only as the home to myriad plant and animal species and as the source of valuable products, but also because they support diverse human cultures. Forests provide shelter and sanctuary for wildlife and they play an important role in the ecology of watersheds. Loss of forests results in severe ecological and economic costs — low watershed protection, local climate change, reduced supply of timber, fuelwood, fodder, fruits, etc. and also affects people's lives.

## PROTECTION AND CONSERVATION OF BIODIVERSITY

**Identification and Long-term Conservation of Ecosystems:** Selection of ecosystems for purposes of *in situ* conservation has to be done with utmost thought and care by following some ground rules. A detailed survey has to be made of specific ecosystems in a country before these are established as protected areas. For each ecosystem that is to be conserved, an inventory should be available of its content of plants, animals and microorganisms.

Most organisations dealing with biodiversity have advocated conservation and enrichment of ecosystems as a whole. Many species can be conserved under *in situ* conditions in just one attempt. This is particularly advantageous in tropical rain forests where many species occur in low densities and have a high degree of endemism.

Some advantages are : (a) Natural selection, co-evolution (with plant diseases and insect pests) and evolution of new taxa would continue unabated; (b) The process would be continuing, dynamic and holistic, (c) Cost-wise, it would be far less expensive than *ex situ* conservation, (d) People's participation and their stake would be ensured, (e) Biosphere Reserve.

## BIOSPHERE RESERVE

The concept of **biosphere reserves** as a method of sustainable development was launched in the early 1970s by the UNESCO Man and the Biosphere Programme (MAB). In contrast to typical national parks or other nature reserves, the biosphere reserve concept allows human habitation within a reserve. Rather than isolating natural

areas and protecting them from humans, biosphere reserves carefully incorporate, limited and sustainable human activities into the planning and management of the area.

In contrast to many natural areas that are selected on the basis of spectacular aesthetics or species of special interest, a biosphere reserve must represent a typical, biotically important terrestrial or coastal site. A goal of the MAB programme is to include representatives of all 193 identified biogeographic provinces of the earth in a biosphere reserve system (Tanglely, 1988). Nearly 500 biosphere reserves exist worldwide today.

According to one of the creators of the concept, Michel Batissee (1966), biosphere reserve should meet three interacting goals : conservation, training and development. The conservation role is typical : reserves should encompass areas large enough to ensure maintenance of genetic, species, habitat, and ecosystem diversity over time. The training role includes education, research, and information exchange, done in a carefully planned manner so that there are no significant perturbations to the reserve.

Three roles of a biosphere reserve are realized by the designation of three geographic regions with a reserve. A **core zone** is a natural, protected area in which only nondisruptive research, such as environmental monitoring, is allowed. Often core zones are pre-existing protected areas such as national parks or nature preserves. A **buffer zone** surrounds or adjoins the cores, and only activities compatible with the protection and preservation of the cores are allowed there. Examples include natural history tourism, education and training low-impact manipulative research, and traditional land uses such as low-intensity agriculture or extraction of renewable natural products. Both of these zones should have clearly defined borders and legal protection. Beyond the buffer zone is a **transition zone**, which has no clear limits. It includes human settlements and economic activities compatible with conservation and preservation of the reserve eco-systems.

**Domestication of Wild economic Biota :** Methods of domestication of wild economic animals and cultivation of wild economic plants will have to be evolved in selected cases. Collecting of some herbal drugs (e.g. aconites, coptis, gugul, etc.) from natural habitats in India is an age-old vocation, but these can be produced easily and on a large scale by simple techniques using seed biology, cuttings and even cloning through tissue culture. This is true of all developing countries.

In order to give a fillip to conservation of biodiversity, some of the wastelands need to be "domesticated" and developed as man-made wilderness areas. Experience has shown that it is possible to attract small mammals, birds, reptiles, insects, micro- and mesoflora and fauna to such areas within a few years of rehabilitation/ restoration. Often such areas are ultimately taken over by natural vegetation.

**Management of Protected Areas And Habitat Restoration :** Most Governmental measures aimed at conservation invariably involve regulations or restrictions on the use of land/forests/other natural resources by the neighbouring human populations. Very often, such efforts fail in the face of antagonism mobilised by conservation measures, which is particular frequently involve projects that have no

evident relevance to the local inhabitants but at the same time require them to forego certain amenities on rights for which they are not compensated.

**Wildlife Protection in Multiple-use Areas :** These areas include worked forests, pastures and grazing lands on the peripheries of protected areas. While meant to serve community needs, these areas also happen to be important wildlife habitats, and in a number of cases provide "corridor" or migration and dispersal pathways for the wildlife of the protected areas they encompass. These areas are especially vulnerable to wildlife poaching, theft of wood and timber and other plant materials, encroachment and settlement, and overgrazing. In such context, NGOs could liaise between Government and the local community to help demarcate the best multiple-use area, suggest the right kind of concessions and help remove the community's sole dependence on local forest resources by popularising alternate technologies for the area — biogas, solar devices, fuel-wood plantations, high-efficiency wood-burning stoves, etc.

**Rehabilitation of Endangered and Threatened Species :** The assessment of endangered species is still far from complete or up-to-date. This work, which is the responsibility of various governmental organisations, need to be greatly strengthened and accelerated. While this time-consuming work is in progress, specific measures must be taken to identify, evaluate ecologically and economically, and re store/restock/ reintroduce certain species in some cases.

**Linkage Between Insitu and Exsitu Conservation :** PAN along with botanical gardens, arboreta, zoos, zoological parks and aquaria, and a variety of biobanks dealing with genes (DNA), sperms, pollen, eggs, organs, tissues and seeds — all need to be organised into a grid under some authority which should oversee and monitor such a network. Such a system must establish meaningful cross-linkages between *in situ* and *ex situ* conservations, which at present follow independent courses. Such cross-linkages would be mutually supportive and beneficial.

**Wildlife Education and Interpretation :** There is now widespread realisation and acceptance of the fact that public awareness of and support to conservation is vital, if the Government efforts in this direction are to succeed and gain adequate momentum. Wildlife education needs to be directed to (i) Politicians, decision-makers and adminis-trators, (ii) Communities living in and around wildlife areas and directly dependent on their resources and (iii) the general public, including students of all levels.

**Domestic Legislation and International Conventions :** Wildlife Laws, forest laws and other regulations, which have an important bearing on the status of wildlife habitats and on wildlife species, need to be reviewed in the light of changing local, national and global circumstances and trends. It is true that if any country's biodiversity is to be saved, maximum public support and participation are essential. We start with the group that pioneered the Chipko Movement, which originated in Garhwal in the hills of Uttar Pradesh.

The Dashauli Gram Swarajya Mandal (DGSM) pioneered the Chipko Movement while one wing of Chipko, identified with Sundarlal Bahuguna has preferred to connect Himalayan deforestation with national and global environmental concerns. DGSM under the leadership of Chandi Prasad Bhatt, has turned from struggle to reconstruction work at the grass root levels. Over the last decade the DGSM has

concentrated chiefly afforestation work in the villages of the upper Alakananda Valley.

Vast areas in the West Bengal region harboured natural Sal forests. Continued pressure on forests, particularly for fuel and fodder as well as means of livelihood, reduced the entire forest area to degraded Sal bushes by the mid sixties. The conventional management practices pursued by the Forest Department were grossly inadequate. The local communities faced acute shortage not only of fuel, fodder and other non-timber forest product, but also of drinking water. At this juncture, Dr. A.K. Banerjee, then DFO of Midnapore District, launched a most innovative and progressive project, called the Socio-economic Project, in a cluster of 11 villages in the Arabari Block of the district. This involved eliciting local villagers in the protection of regenerating Sal forests through the formation of forest protection committees (FPC) in return for free usufructs of all non-timber forest produce (NTFP), first preference for employment and a promise of 25% share in the sale proceeds of short rotation Sal poles. There are 618 families participated in the project protecting 1272 ha of forests. Based on the overwhelming success of the Arabari Pilot Project, the joint management of forest lands programme gradually spread to neighbouring areas through the initiative of both the forest department and the people themselves.

In the Rio Convention, it was internationally agreed upon by the participating leaders of the different countries to safeguard the common concern of humanity by conserving nature and utilising its genetic resources in sustainable manner. Conservation programmes are accordingly implemented at three levels, namely genetic, species and ecosystems. Different approaches are adopted for this purpose, especially *in situ* and *ex situ* conservation methods.

*In situ* conservation of wild flora through protection of species habitats and ecosystems is chiefly being implemented by the Ministry of Environment and Forests. *Ex situ* conservation of genetic resources of cultivated plants and their wild relatives is managed and maintained largely by the National Bureau of Plant Genetic Resources under the Indian Council of Agricultural Research. Moreover the non-governmental organisations (NGOs) play a pivotal role to make people's participation possible to protect and conserve biological diversity. At the international level, six major organisations have been interested in conservation and utilisation of biodiversity. These include United Nations Educational Scientific and Cultural Organisation (UNESCO), Food and Agricultural Organisation (FAO), United Nations Environments Programme (UNEP), United Nations Development Programme (UNDP), International Board of Plant Genetic Resources (IBPGR) and other institutes under the Consultative Group on International agricultural Research (CGIAR), World Resources Institute (WRI), International Union for Conservation of Nature and Natural Resources (IUCN), now renamed as World Conservation Union (WCU), and Worldwide Fund for Nature (WWF), earlier known as World Wildlife Fund. Out of these, UNESCO has contributed substantially in building up a conceptual and philosophical framework through its Man and Biosphere (MAB) and other programmes, such as the network of *in situ* conservation areas like Biosphere reserves, National Parks and Sanctuaries.

Increase in demands of food, fuel, fibres and shelter by the ever-increasing human population has considerably eroded the natural resources. The single most affected resource is biodiversity. Since biodiversity represents the genepool in various

living beings, and it provides the raw materials for plant breeding, its devastation would have catastrophic effects on the survival of the human race. Keeping this in mind, the conservation of biodiversity is a must for the prosperity of mankind.

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# Mushroom Cultivation

PROF. N. SAMAJPATI

## **S**PAWN SUBSTRATES

A number of materials, mostly agricultural wastes, whether alone or in combination, can be used to prepare mushroom spawns. These vary from region to region and usually depend on the availability of the substrates. Those that are being used, to mention a few, are chopped rice straw, tobacco midribs, sawdust, water hyacinth leaves, cereal grains, used tea leaves, cotton waste, cotton pulp and husks, lotus seed husks, etc. The most important aspect to consider is good growth of the mushroom mycelium on the given substrates. Availability and ease of preparation should also be considered. In most laboratories, cereal grains sorghum, rice, wheat or rye are used as mother spawn, and agricultural wastes as the planting spawn substrate.

## SPAWN CONTAINERS

Different containers are used in preparing spawns, and these likewise vary from region to region. The most important requirement is that it should be heat resistant plastic bags, polypropylene, are also now popularly used due to their light weight in handling and transport. Milk bottles and wide-mouth jars are also commonly used. An average size is 500 to 1000 ml capacity. Bigger containers would be difficult to sterilize. Container cost should also be considered so that it will not add much to the cost of the spawn. Dextrose bottles which can be obtained free, in most hospitals, are ideal because they have rubber stoppers with air outlets that can be loosely plugged with cotton. They have to be broken, however, by the planters to get the spawn inside and this is rather risky.

## BASIC PREPARATION TECHNOLOGIES

**Preparation of Master or Grain Spawn :** The so-called roaster spawn is used to inoculate spawn bags to be used as planting materials for growing mushrooms. In the case of *Pleurotus*, *Auricularia* or *Lentinus*, the master spawn is used to inoculate the actual fruiting bags. To prepare grain master spawn, wash the seeds wheat, rye, sorghum or rice thoroughly, then soak overnight. Remove dead seeds or those that float on water. The following day, wash the seeds again, then boil in water till slightly expanded at least 10 to 15 minutes. Cool while draining. Pack them loosely on flat or round bottles that can withstand intense heat. Fill up to about 2/3 of the bottle, then

plug with cotton or similar plugs. Sterilize the bottles in a pressure cooker for about 1 hour at 16 lbs pressure. Steaming for 2 hours in a big cast iron casserole may also be done instead. Let cool before inoculation with previously prepared agar mycelium.

Stawdust may also be used instead of grains. In this case, rice bran at 20%, is mixed with dry sawdust. Sprinkle enough tap water to make 45% moisture content (this is determined by pressing a handful of the mixture and if no water runs off in between the fingers and the materials stay in form after releasing the pressure, then you have more or less 45% moisture in the mixture). Fill flat or round bottles up to the neck. Tap the bottle to make the mixture compact. Clean the mouth and plug with cotton. Sterilize under pressure for one hour or steaming for 2 hrs. Inoculate when cool.

**Preparation of Planting Spawns :** As mentioned above, planting spawns are those that are used in actual mushroom cultivation. These are the ones that are usually sold or distributed to growers. In the case of the plastic bag technology of growing *Pleurotus*, *Auricularia* and *Lentinus*, planting spawns are usually eliminated. The master spawn are inoculated directly to the fruiting bags.

For *Volvariella*, coirdust mixed with dried ipil leaves is commonly used as spawn substrate. Coirdust is a waste from the coconut factory, actually husk that has been ground like sawdust. This is easier to use because there is no need for chopping as in the case of rice straw or dried tobacco midrib. In Hongkong tea leaves waste are used, while lotus seed husks are commonly used in Thailand.

Other substrates that can be used are dried water lily plants, dried banana leaves, dried grasses and other leaves, all previously dried, chopped or cut into small pieces. They are soaked in clean water overnight, then drained or squeezed. They may also be provided with food supplements, such as dried ipil leaves, rice bran or corn meal at 5:1 ratio. Mix the mixture thoroughly, fill up in bottles or bags and sterilize as in *Pleurotus* and *Auricularia* and *Lentinus* fruiting bags.

For the log technology of growing *Auricularia* and *Lentinus*, the same saw-dust mixture used for master spawn, is used as the planting spawn.

For *Agaricus* spp, cereal grains is the most commonly used substrate, prepared the same way as the master grain spawn. The manner however of preparing the spawn is primarily a matter of personal or institutional preference. Some spawn growers prefer that they call bulk operation where the sterilized grains are inoculated in bulk, then semiautomatically and aseptically transferred from a large rotary blender to sterile polyethylene (ordinary) plastic bags, where mycelial colonization of the substrate occurs. Big spawn making plants in US and Europe use advanced clean room and sterile air techniques and facilities to prepare spawns in big quantities.

## MAINTENANCE OF SPAWN QUALITIES

A major source of contamination of growing mushroom spawn is the grain seed or the substrates used to prepare the spawn. Modern equipment and facilities for sterilization and maintenance of sterile conditions are capable of reducing fungal and bacterial contamination to 0.1% of the number of spawn units. Quality control in spawn making consists of constant inspections to eliminate those visibly

contaminated or exhibiting unacceptable differences in appearance and growth which are signs of degeneration and mutation.

Spawns of most mushrooms can be refrigerated but they should be back to normal room temperature and to its active state before they are used to inoculate planting spawns or used as planting spawns. At room temperature, the spawns should not be more than 4 weeks after filling up. The presence of pink chlamydospores in *Volvariella* spawns indicates a highly fertile and active growth.

Active growth of the planting spawn is a requisite to good growth and yield. In the case of *Volvariella* outdoor cultivation, planting substrates are not presterilized. Therefore, the spawn will compete with the naturally present organisms on the planting substrates. If the spawn is not active, the mushroom mycelium will be overgrown by these organisms. If it is active, it will overgrow these unnecessary organisms and produce more yield. The same is true for other unsterilized substrates such as for shiitake and *Auricularia* logs.

## SPAWN QUANTITIES

Quantity or amount of the spawn does not directly affect yield but it can affect yield indirectly by eliminating the unnecessary organisms present in the planting substrates. The more the spawn, the faster will it overgrow the substrate and hinder grow of contaminants, hence yield will be regular and not be affected by these contaminants.

For *Lentinus* log cultivation, one flat bottle of spawn will be enough to inoculate 4 to 5 one meter-long billet or oak log. Each log can produce a minimum total yield of 1 k fresh shiitake in its entire cropping life of 2 to 3 years, 2 croppings per year.

For *Pleurotus* cultivation, one bottle of grain or sawdust spawn can inoculate 40 bags. Each bag weighing 40 g of rice straw mixture from an original dry weight of 125 g should yield at least 200 grams of fresh mushroom or 200% biological efficiency in its 3 months cropping life. used

For *Agaricus*, one liter grain spawn is usually used to plant every square meter area of the bed. While the average yield varies according to the compost composition.

## VOLVARIELLA

### OUTDOOR TECHNOLOGY

**Construction of Soil Base :** Soil bases should be elevated for at least 6 inches from the ground level to facilitate drainage. This should be at least 18 inches or 1-1/2 feet wide and 12 feet long or 2-3 meters. The elevation can be made by excavating the soil around the bed, thus constructing a ditch around the bed that can be flooded and used as source of water as well as of humidity. These bed foundation can also be cemented or constructed with wood if so desired. Or simply clean levelled bed are thoroughly without raising it and mark it so you know where to lay the substrates.

It may also be desirable to construct nipa roofs or shed to cover and protect the bed from excessive sun or sudden rain. The beds can also be built under vegetable trellises.

**Bed materials :** Rice straw is the most commonly used bedding for the mushroom. These should be relatively clean and well-dried. Decayed straws harbor destructive organisms especially *Coprinus* spores from the air that will hinder production. Dried banana leaves still hanging on the banana plants can also be used as well as dried waterhyacinth plants. Whole waterhyacinth plant is used including the roots. Remove excess soil from the roots after pulling from the water, then dry in a clean place (preferably on a cemented area) of hanging, for at least one week.

The straw should be arranged and tied in bundles. With a bolo, cut them at 2 ends in uniform length of around 1-1/2 feet (same as the width of the bed foundation). The bundles should at least be 4 inches in diameter. Do the same with dried banana leaves and the waterhyacinth plants. Four or 6 plants laid parallel together and folded at ends can constitute one bundle. Trim at both ends as done with rice straw.

**Preparation of bed materials :** Soak the bundles in a clean water tank or drum for a least 2-3 hours, even 24 hours. Drain the bundles for a few minutes, then pile neatly on the soil or cemented base compactly side by side perpendicular to the width of the base. The soil or foundation should be first thoroughly wet so as not to absorb the water from the bundles. Two poles or sticks are poked at 2 ends to hold the bundles in place.

**Spawning :** After one layer is complete, bits of spawn (thumb size) are placed on top of the layer about 2-3 inches from the edge and 4 inches apart. Place the remaining layer or bundles, pack real well, then repeat spawning of every layer made. As soon as the piles are complete (around 4-5 piles or layers), cover the bed with the remaining straw. During summer, the bed should have only 4-5 layers or at least 1-1/2 feet high so the bed will not heat very well. More layers or higher beds may be constructed during cooler season to provide enough heat (at least upto 35°C) for rapid development of the mycelium. The edge of the bed should be even as much as possible so that harvesting would be easy. If it is very irregular, mushrooms will come out on deep places or grooves on the edge and it would be difficult to be seen, hence they will just rot there. Mushrooms need air and some light, so they will come out on the edge during fruiting. Trimming loose stands with big scissors may be done after spawning and before covering, to provide neat edge.

**Maintenance :** A plastic sheet may be used to cover the bed for the first 5-6 days. This will bring about the right temperature of 35-38°C for the growth of the spawn unto the rice straw. This will also keep the moisture so that there is no need to water the bed during the first 10 days. In hot weather the beds should be only loosely covered and/or openings should be provided on the top portion of the beds.

A bamboo foundation lined with plastic may also be used so that the plastic will not be touching the straws. This "tent" can be used until harvesting. This foundation may be lined with straw or nipa or grasses to provide shade at the same time.

The canal along the bed if present may be filled with water in order to maintain the right humidity and also to prevent insects from creeping into the bed.

**Fruiting :** Eight to 10 days after spawning, the pinheads or small white fruiting bodies should begin to appear. Lift the plastic for a few minutes to provide good ventilation. Do not water anymore. It will take another 2 to 3 days from the appearance of these minute fruiting bodies, for the mushroom to be ready for harvesting.

The mushrooms are best harvested at the button stage. Mushrooms must be picked at least twice a day for the next 3 days. There are often mushrooms at different stages of development growing so close together that care needs to be exercised during harvesting to prevent damage to the developing or very young mushrooms.

The first 3 days flush is followed by the next flush after a rest period of 5-7 days. This will continue for a period of at least 1 to 2 months.

A 3-m bed requires at least 150-200 bundles of rice straw or 25 Kgs. dried rice straws. It needs around 6 bottles of spawns (500 ml dextrose bottles) and is expected to yield at least 6 kilos of buttons

## PLEUROTUS

*Pleurotus* mushroom is one of the edible mushrooms that can be cultivated in the tropics, although it is more sub-tropical. Like the other mushrooms, it contains a lot of protein, minerals and vitamins which are essential for the maintenance of good health. It is also cultivated in Europe where it is known as oyster mushroom (*P. ostreatus*), and in China, where they call it abalone mushroom (*P. abalonus* or *P. cytidiosus*). Several other species are now available for cultivation. There is *P. sajor-caju* (originally from India), *P. florida* (probably a different strain of *P. ostreatus*) and *P. flabellatus* (from the Philippines).

The above species of *Pleurotus* are suited for growing in conditions ranging from 15°C to 32°C. *P. sajor-caju* has been tested to be most resistant to the tropical climate of 28-32°C, although they still fruit faster and bigger at 22-26°C during the cooler months of the year. *P. abalonus* prefers lower temperatures of 22-26°C while *P. ostreatus* are the so-called low temperature *Pleurotus*, fruiting mostly at 15-20°C, hence suited to the temperate climate of Europe and USA.

Like the other mushrooms, *Pleurotus* can be grown in various ways using various agricultural waste materials. It can be grown in a mixture of saw dust and rice bran, rice straw and bran, sawdust and ipil leaves, and other various combinations. Other wastes like corn cobs, cotton hulls, sugarcane bagasse and leaves, corn leaves, grasses, rice hull, waterlily leaves and others are also good substrates for growing this mushroom.

Similarly, it can be grown on a variety of containers like plastic bags, native baskets, jars, plastic trays, shelf beds, etc.

The equipments that are used in the process also vary according to resources. In big commercial scale, a pressure cooker or a casserole is not feasible. Big steamers, sometimes one room steamers, are used. For home industry, a big

casserole or a drum can be utilized in the sterilization of the mushroom bags or container. Latest findings show that in cool places, sterilization is not necessary. In fact it can be grown on substrate/beds without sterilization especially during cool season (20-25°C).

## COMMERCIAL PRODUCTION

**Materials or Substrates that can be used :** The following can be used to grow the mushroom : rice straw, dried water hyacinth, banana leaves, cotton wastes or sawdust. To ensure good results, these materials are usually composted for 6 days before use (30 days for sawdust).

To compost rice straw, follow the same formula and method as for *Volvariella* but urea may be omitted. Cotton wastes and water hyacinth, begin fast decomposing, are composted for only 3-4 days. After composting, add 20% rice bran and again 1% lime. Cotton wastes and rice straw may also be combined and added with rice bran to a richer substrate combination.

To compost sawdust, pile it into a heap not higher than 1.5m after combining urea and lime at both 1% combination. Wet thoroughly before piling then cover with plastic sheets. During fermentation, successive turnings have to be done every 7 days over the entire 30-40 day period. By that time, the sawdust mixture will become soft and no rancid or bad smell. If the compost will not be used right away, this should be dried before being stored.

When the mixture is ready, add 10% rice bran, then fill up 6 x 12 polypropylene bags, with the mixture. With the use of your fist, punch the compost down so as to make it quite compact. Then insert a cut PVC pipe (2 inches dia and 1 inch thick) into the mouth of the bag, pulling it up to tighten, then fold over the plastic over the PVC bottle neck to provide an opening for inoculation. This is then plugged with cotton that fits in the opening. This plug is covered with a piece of paper held in place by a rubber band.

The use of composted or uncomposted substrates varies according to personal preference. To use uncomposted straw, soak it overnight, drain, then add 50% rice bran before bagging. To use uncomposted saw dust, increase rice bran also to 50% of saw dust.

**Sterilization :** Nowadays, use of pressure cooker is not recommended anymore because sterilization by pressure not only kills all the microorganisms present in the mixture but it also destroys certain substrates in the compost which inhibit or control spore germination. Likewise, food in the compost are broken down into forms favorable for the needs of the contaminating microorganisms. Thus those that are sterilized by pressure are easily contaminated.

Sterilization by steaming at 100°C is now more acceptable because besides the cost is lower (the steamer can only be an ordinary big capacity casseole or a drum),

bags thus steamed are less susceptible to contamination. Steam the bags for 1-2 hrs depending on the substrates and the volume.

**Inoculation / Spawning :** Since you are using sterilized substrates, spawning should be done in an aseptic place, preferably in the same inoculation room used in tissue culturing, or inoculation of spawn substrates. Unless this is done in a commercial scale, any clean area of the house may also be used. Spray the surrounding thoroughly with plain water or 10% chlorox solution.

Grain or sawdust spawns are used. For grain spawn, the bottle is shaken to separate the seeds, then after removing the plug and flaming the mouth of the bottle, some grains are poured into the compost bag. Return both the plug of the spawn and the plug of the compost bag and proceed to the next bag. The newly inoculated bag is slightly tilted to dispense equally the few grains in the shoulder area of the bag around the neck.

For sawdust spawn, a piece of the spawn is either picked up by a long flat spooned needle especially designed to scoop the spawn or the spawn is stirred up with needle then poured off as with grains. One bottle of grain spawn in 500-ml dextrose bottle is enough to inoculate 50-60 bags. One bottle or bag of sawdust spawn can be used for 50-100 ml bags.

**Incubation :** The spawned compost bags are then kept preferably in a darkroom until the mycelium have fully penetrated the substrate downwards. In case no white growth appears in 5 days, then perhaps the spawn used is already dead or the substrate is too dry or it is contaminated with other spored organisms. If contaminating fungi (usually black or green) appear on the surface, then contamination occurred during inoculation. If these contaminants are all over the substrate, then the substrate has not been thoroughly sterilized.

In 20-30 days depending upon the substrate/substrate combination, the bags will be fully filled with white mycelium. This means it is time to open the bags for fruiting initiation. Wait for 1-2 more weeks before opening to make sure the mycelium is mature enough to fruit.

**Fruiting :** A mushroom house, the size of which will depend on the number of bags prepared at a time, should be provided. This house may be made up of nipa or sawali or wooden concrete walls and similar roof. There should be air vents on the upper walls to facilitate aeration badly needed for the developing fruiting bodies, at the same time providing light inside the house. If drafty, the walls may be covered with plastic sheets to provide the much needed humidity (80-85%) of the mushrooms.

Shelves made up of bamboo or wood line up both sides of the house with a center aisle. The shelves are on bamboo frames, one shelf above the other with about 45 cm space between them.

The matured bags are first opened by removing the plug and the PVR pipe neck then rolling down the mouth of the bag. Otherwise, the mouth portion is cut off with a razor blade or the bag may just be slit either criss-crosses at 4-6 places or simply slashed lengthwise. When following this latter technique, the bags may be hung with

rope or string.

Fruiting requires right temperature (20-28°C), ventilation, little light, and enough moisture and humidity. To provide moisture, daily watering of the house is required. Watering should not be so excessive to avoid rotting of bags most especially if water retains inside the opened bag.

If temperature inside the house is more than 30°C, spray frequently to lower the temperature and hasten fruiting. Doors and windows may also be opened especially at night to let the cool air of the night come in.

If the mushrooms develop long stalks and small caps, then it is an indication of poor ventilation inside the house. It may also be possible that there is not enough light inside.

**Harvesting :** Three to 4 days after opening the bags, mushroom primordia will start to form and these will be ready for harvesting in another 2 more days. If the bag is not mature enough, it will take longer time to fruiting.

When harvesting, grasp the stalk of the mushroom and gently pull it out. Do not use knife. If kept in the refrigerator or a cool place, it will stay fresh up to 3-6 days.

**Increasing Yield Maintenance :** The most common fertilizer is urea – added with water (100 gm in 100 liters water). Use mist plastic sprayer and spray directly on the surface.

After harvesting, turn the bags on the other side and this time, open the bottom portion. After harvesting from this portion, slit the middle so that mushrooms will come out. As long as the substrate appears white, fruits can still be expected. When the mycelium appears colorless, soft and dead, it is then time to get the bags out of the house.

Yield is about 100-200% of the dry weight of the bag. It depends on the substrate combination as well as the way the bags are maintained during fruiting season. From our observation, the richer the combination, the whiter and denser the mycelium, and the more fruits expected.

## AGARICUS

*Agaricus* mushrooms are better known as the "champignon", the button mushrooms, the French or the Western mushrooms. They were first cultivated in caves in France sometime in the seventeenth century and spread to other parts of the world two decades after. Progress from the primitive culture to the more highly sophisticated technologies are results of research conducted in USA and many countries of Europe and Asia. The highly technical controlled methods of growing the mushrooms had contributed to the worldwide growth of *Agaricus* world production which accounts to 74% of the total world production and demand for cultivated mushrooms.

While these developed countries are blessed with those scientific and engineering facilities that had made mushroom growing nearly independent of outside climatic conditions, growers in less fortunate countries had however taken steps to improvise and provide whatever is needed for maximum yield at right environment. The method that will be described here is that basically followed in some countries of Asia, using the minimal basic requirements for growing the mushroom.

**Substrate Preparation/Raw Materials Needed :** The grower has a wide choice of raw materials or compost formulations that could be used in growing these mushrooms. The choice depends upon the availability of the raw materials and the favourable reaction of the particular strain being used, to the mixture or the materials. Some of the commonly used and recommended are :

#### **Philippines**

Rice straw	100.0 k
Chicken manure	20.0 k
Ammonium nitrate	1.0 k
Calcium sulphate	2.5 k
Lime	5.0 k
Rice bran	10.0 k

#### **Thailand**

Rice straw	100.0 k
Urea	1.0 k
Ammonium sulphate	0.5 k
Muriate of potash	1.2 k
Double super-phosphate	1.2 k
Gypsum	2.5 k
Ricebran	1.0k

#### **Tiwan (in commercial)**

Rice straw	30.3 k
Ammonium sulfate	0.16 k
Urea	0.15 k
Superphosphate	0.6 k
Calcium carbonate	0.9 k

#### **India**

Rice straw	300.0 k
Rice bran	15.0 k

	Calcium ammoniumnitrate	9.0 k
	Urea	3.0 k
	Superphosphate	3.0 k
	Potassium sulphate	3.0 k
	Molasses	5.0 k
	Gypsum	30.0 k
	Gamma BHC	69 ml
	Temik 10 G	120 g
<b>Korea</b>		
	Rice straw	30.3 k/m
	Ammonium sulfate	0.6
	Urea	0.15
	Superphosphate	0.6
	Calcium carbonate	0.9

**Composting :** Composting is usually done under a shed for protection against rain and too much heat. The floor of the composting site should be concrete to facilitate cleaning operations.

The straw is first cut to about 5 inches long, then mixed with the other materials to form a heap of about 3 to 5 ft. high. The heap is wetted thoroughly with water and left to ferment for 4 to 7 days, never allowing it to dry up by covering with a big plastic sheet. On the 7th day of heating up, the pile is now turned and this is done for at least 3 to 4 days interval until 28 days during which time, the compost turns dark in color and becomes homogenous, partially broken down and soft. There should be no ammonial smell left.

The partially decomposed materials are now placed in wooden trays of 6 to 8 inches deep and further decomposed in a room where steam is injected for 2 to 4 hours until a bed temperature of 60°C is attained. This pasteurization will kill all insect pests and fungal competitors. The temperature should be maintained for 15 to 18 hours. After this, the finished compost is allowed to cool to around 45° to 50°C for 2 to 3 days. Then air is introduced to the room by a blower until the compost is around 30°C, ready for spawning. This indoor pasteurization may be omitted when growing in small scale. Simply fill the previously disinfected boxes or shelves with the compost and ready for spawning.

**Spawning :** The spawn should be carefully broken into pieces and inserted 2 inches deep at intervals of 9 to 10 inches on the bed. If grain spawn is used, the spawn bottle is simply shaken up to separate the grains, then sprinkling a given quantity over the surface of the bed is done. The surface is then covered with sheets of paper to

conserve moisture. After spawning, the beds are further incubated in the same or another room at 22 to 24°C for 2 to 3 weeks, during which time, the organism is expected to grow through the substratum.

Care is taken so that the beds are never allowed to dry. Too much water however will kill the growing mycelium.

**Casing :** Casing materials may be composed of garden soil or a mixture of soil and peat. The pH should be around 5.5 to 7.8. The casing soil should be previously pasteurized for at least 4 hours. They may be stored tightly in closed plastic bags for 6 to 12 months before use.

To case, sprinkle evenly on the surface covering the entire surface with at least 5 cm deep of the casing material. Then sprinkle with just enough water. Continue incubation for 7 to 10 days at 18 to 24°C, depending on the strain or species. Never allow the surface to get dry or too wet.

**Fruit Initiation and Development :** Ten days after casing, lower the temperature to about 14 to 18°C if cooling system is available to allow fresh air to come in and clean off the gas excreted by the mycelium during the previous incubation period. Mushroom primordia will now appear on the surface for the next 6 to 12 days. Continue incubation and ventilation throughout the development of the mushrooms. Too high concentration of CO<sub>2</sub> (insufficient ventilation) inhibits primordia and causes abnormality in the resulting mushroom. Average yield reported is about 40 k per 3 sq. meter bed area.

In case cooling system is not available, schedule the planting so that fruiting time which usually occurs at least one month after spawning occurs during the time when the weather outside and inside the house is 12 to 18°C.

# Bio - Ethics : Some Major Concerns

DR. AMIT KRISHNA DE

## E THICS IN HINDU PHILOSOPHY

The Hindu Philosophy emphasises the importance of all forms of bios as being having spiritual dimensions. The Hindu text stress the great rhythms of nature and believes that divine power expresses through animals, plants and human beings. Man is looked upon as a part of nature, part of bio-environment. The forests and groves are regarded as sacred. Humanity should treat animals with sympathy and ahimsa.

### MAIN GOAL

The basic goals in understanding issues related to bioethics are :

(i) To sensitise the people on the multifaceted ethical issues created in modern society as a results of technological progress, (ii) To investigate the ethical factor into all kinds of scientific endeavour (iii) To bring out the importance of bio-technology for the progress of human civilisation and the maintenance and promotion of bios and (iv) To inform people not only of the achievements of Science and Technology but also of their difficulties, problems and limitations.

### BIO-RIGHTS

The bio-rights as related to Bio-ethics involves - i) The Right to live, to exist on Earth, to give birth to the progeny, (ii) Satisfaction of the needs of different forms of life, the improvement of their quality of life and (iii) Preservation and the further enhancement of bio-diversity.

- **The Human Rights** : any human being must be granted the right to enjoy living in contact with multifaceted bios. Humanity should also have the right along with all other forms of bios to be protected against destructive effects caused in the global environment such as ultraviolet radiation by an ozone screen.
- **The Animal Rights** : cruel treatment with animals, part of the laboratory routine in the past was eradicated in most laboratoris of the world after introduction of new legislation on experiments with animals in the 70's and 80's. Detailed protocols concerning human rules of animal research were developed worldwide. Numerous powerful movements of animal's friend and defenders of animal rights took shape in different countries.

- **The Plant Rights** : plants serve as reliable bio-indicators of heavy metal contamination and are a source of medicine for human welfare. The oxygen released by it is vital for all the other forms of life. The enhancement of plant rights can help humanity overcome the ozone hole problem.
- **The Microbial Rights** : the micro-organisms functions in food and feed production, in plant protection against insects and weeds, in energy production and depollution of environment. Advances in biotechnology has produced bio-factories with immense potentiality for benefit of human welfare. However, patents in microbial life is an ethical issue.

## CURRENT ETHICAL CONCERNS

### Animal Research and Bioethics

To end all human "exploitation" of animals - this includes, but is not limited to, raising and slaughtering of livestock for human or animal consumption, eating meat, hunting, using animals for any medical or veterinary research, zoos, circuses, rodeos, horseshows, dogshows, animals performing in TV commercials, shows or movies, guide-dogs for the blind, police dogs, search and rescue dogs, and the practice of owning pets.

Some areas where ethical questions have been raised are

- Should the notion of animal rights be confined to mammals or should it extend to all vertebrates, insects, worms and protozoans?
- Should sacrifice of animals for purposes other than scientific research be encouraged?
- Should a distinction be drawn between pharmacological and bio-medical studies where animals appear indispensable for testing?
- For medicine and fundamental research where alternatives may be available? eg. LD<sub>50</sub> studies.

Research should be undertaken with a clear scientific purpose. There should be a reasonable expectation that the research will (a) increase knowledge of the processes underlying the evolution, development, maintenance, alteration, control, or biological significance of behavior; b) determine the replicability and generality of prior research; c) increase understanding of the species under study; or d) provide results that benefit the health or welfare of humans or other animals. The scientific purpose of the research should be of sufficient potential significance to justify the use of animals. Researchers should act on the assumption that procedures that would produce pain in humans will also do so in other animals. The species chosen for study should be best suited to answer the question(s) posed. The facilities housing animals should meet or exceed current regulations and guidelines. All procedures carried out on animals are to be reviewed to ensure that the procedures are appropriate and humane. Animals are to be provided with human care and healthful conditions during their stay in the facility.

Human consideration for the well-being of the animal should be incorporated into the design and conduct of all procedures involving animals, while keeping in mind the primary goal of experimental procedures - the acquisition of sound, replicable data. Behavioral studies that involve no aversive stimulation to, or overt sign of distress from, the animal are acceptable. These include observational and other noninvasive forms of data collection. When alternative behavioral procedures are available, those that minimize discomfort to the animal should be used. Whenever consistent with the goals of the research, consideration should be given to providing the animals with control of the potentially aversive stimulation. Procedures in which the animal is anesthetized and insensitive to pain throughout the procedure and is euthanized before regaining consciousness are generally acceptable. Procedures involving more than momentary or slight aversive stimulation, which is not relieved by medication or other acceptable methods, should be undertaken only when the objectives of the research cannot be achieved by other methods. Experimental procedures that require prolonged aversive conditions or produce tissue damage or metabolic disturbances require greater justification and surveillance. These include prolonged exposure to extreme environmental conditions, experimentally induced prey killing, or infliction of physical trauma or tissue damage. An animal observed to be in a state of severe distress or chronic pain that cannot be alleviated and is not essential to the purposes of the research should be euthanized immediately.

Procedures involving the use of paralytic agents without reduction in pain sensation require particular prudence and human concern. Use of muscle relaxants or paralytics alone during surgery, without general anesthesia, is unacceptable and should be avoided. Aseptic (Methods that minimize risks of infection) techniques must be used on laboratory animals whenever possible. All surgical procedures and anesthetization should be conducted under the direct supervision of a person who is competent in the use of the procedures. If the surgical procedure is likely to cause greater discomfort than that attending anesthetization, and unless there is specific justification for acting otherwise, animals should be maintained under anesthesia until the procedure is ended. Sound postoperative monitoring and care, which may include the use of analgesics and antibiotics, should be provided to minimize discomfort and to prevent infection and other untoward consequences of the procedure. Animals cannot be subjected to successive surgical procedures unless these are required by the nature of the research, the nature of the surgery, or for the well-being of the animal.

When the use of an animal is no longer required by an experimental protocol or procedure, in order to minimize the number of animals used in research, alternative uses of the animals should be considered. Such uses should be compatible with the goals of research and the welfare of the animal. Care should be taken that such an action does not expose the animal to multiple surgeries. The return of wild-caught animals to the field can carry substantial risks, both to the formerly captive animals and to the ecosystem. Animals reared in the laboratory should not be released because, in most cases, they cannot survive or they may survive by disrupting the natural ecology. When euthanasia appears to be the appropriate alternative, either as a requirement of the research or because it constitutes the most human form of disposition of an animal at the conclusion of the research : Disposal of euthanized animals should be accomplished in a manner that is in accord with all relevant legislation, consistent with

health, environmental, and aesthetic concerns. No animal should be discarded until its death is verified.

**Field Research :** Field research, because of its potential to damage sensitive ecosystems and ethologies, should be subject to certain regulations. Every effort should be made to minimize potential harmful effects of the study on the population and on other plant and animal species in the area. Research conducted in populated areas should be done with respect for the property and privacy of the inhabitants of the area. Particular justification is required for the study of endangered species. Such research on endangered species should not be conducted unless approval has been obtained from suitable committees.

**Educational Use of Animals :** Laboratory exercises as well as classroom demonstrations involving live animals can be valuable as instructional aids. Animals may be used for educational purposes only after review by a committee appropriate to the institution. Some procedures that can be justified for research purposes may not be justified for educational purposes. Consideration should be given to the possibility of using nonanimal alternatives.

### **New Genetics**

**Fairness in the use of genetic information** by insurers, employers, courts, schools, adoption agencies, and the military, among others.

*Who should have access to personal genetic information, and how will it be used?*

**Privacy and confidentiality of genetic information.**

*Who owns and controls genetic information?*

**Psychological impact and stigmatization** due to an individual's genetic differences.

*How does personal genetic information affect and individual and society's perceptions of that individual?*

*How does genomic information affect members of minority communities?*

**Reproductive issues** including adequate informed consent for complex and potentially controversial procedures, use of genetic information in reproductive decision making, and reproductive rights.

*Do healthcare personnel properly counsel parents about the risks and limitations of genetic technology?*

*How reliable and useful is fetal genetic testing?*

*What are the larger societal issues raised by new reproductive technologies?*

**Clinical issues** including the education of doctors and other health service providers, patients and the general public in genetic capabilities, scientific limitations, and social risks; and implementation of standards and quality-control measures in testing procedures.

*How will genetic tests be evaluated and regulated for accuracy, reliability and utility?*

*(Currently there is little regulation at the federal level).*

*How do we prepare healthcare professionals for the new genetics?*

*How do we prepare the public to make informed choices?*

*How do we as a society balance current scientific limitations and social risk with long-term benefits?*

**Uncertainties** associated with gene tests for susceptibilities and complex conditions (e.g., heart disease) linked to multiple genes and gene-environment interactions.

*Should testing be performed when no treatment is available?*

*Should parents have the right to have their minor children tested for adult-onset diseases?*

*Are genetic tests reliable and interpretable by the medical community?*

**Conceptual and philosophical implications** regarding human responsibility, free will vs genetic determination and concepts of health and disease.

*Do people's genes make them behave in a particular way? Can people always control their behavior?*

*What is considered acceptable diversity?*

*Where is the line between medical treatment and enhancement?*

The major **Health and environmental issues.**

*Are GM foods and other products safe to humans and the environment?*

*How will these technologies affect developing nations' dependence on the West?*

**Commercialization of products** including property rights (patents, copyrights and trade secrets) and accessibility of data and materials.

*Who owns genes and other pieces of DNA?*

*Will patenting DNA sequences limit their accessibility and development into useful products?*

### **Human Genome Project**

- In any human population, a great number of individuals are carriers of genes responsible for diseases. Is it moral and justifiable to identify these carriers by authorizing compulsory genetic control of all the citizens of a country?

- Currently, employers are reluctant to engage smokers and corpulent people. Should an employer get access to the genetic data of a person applying for a job?
- Should a person be notified of his being genetically predisposed to a severe, chronic or lethal disease / What if the risk is unavoidable and the disease is incurable? Under what circumstances should a person be allowed to have access to this information?
- Prenatal diagnosis of genetic disorders may become a routine procedure. Whether a pregnancy is to be interrupted if a genetic disease is revealed?
- Knowledge about the genetic make-up of human beings will have a considerable impact on culture. Will it promote the dominance of a purely biochemical vision of man?
- The critical question whether it will be permissible to consciously design human beings?

### **Genetically Modified (GM) Foods**

Although "biotechnology" and "genetic modification" commonly are used interchangeably, GM is a special set of technologies that alter the genetic makeup of such living organisms as animals, plants, or bacteria. Biotechnology, a more general term, refers to using living organisms or their components, such as enzymes, to make products that include wine, cheese, beer, and yogurt. Combining genes from different organisms is known as recombinant DNA technology, and the resulting organism is said to be "genetically modified," "genetically engineered," or "transgenic." GM products (current or in the pipeline) include medicines and vaccines, foods and food ingredients, feeds, and fibers. GM crops are grown commercially or in field trials in over 40 countries and on 6 continents. In 2000, about 109.2 million acres were planted with transgenic crops, the principal ones being herbicide – and insecticide – resistant soybeans, corn, cotton and canola. Other crops grown commercially or field-tested are a sweet potato resistant to a virus that could decimate most of the African harvest, rice with increased iron and vitamins that may alleviate chronic malnutrition in Asian countries and a variety of plants able to survive weather extremes. On the horizon are bananas that produce human vaccines against infectious diseases such as hepatitis B; fish that mature more quickly; fruit and nut trees that yield years earlier and plants that produce new plastics with unique properties.

Technologies for genetically modifying (GM) foods offer dramatic promise for meeting some areas of greatest challenge for the 21st century. Like all new technologies, they also pose some risks, both known and unknown. Controversies surrounding GM foods and crops commonly focus on human and environmental safety, labeling and consumer choice, intellectual property rights, ethics, food security, poverty reduction and environmental conservation.

## Other publications of UGC-ASC, CU

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| 1. <i>Re-readings : Literature and Culture</i>                               | : | Dr. Sanjukta Dasgupta<br>Dr. Tapati Gupta    | Rs. 220/- |
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